# *THE RESPONSES OF* LAGER BREWING YEAST TO LOW TEMPERATURES

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## ABSTRACT

The removal of yeast biomass (cropping) at the end of fermentation to inoculate a subsequent fermentation (serial-repitching) is common practice in the brewing industry. Between successive fermentations cropped yeast is stored as a slurry in cooled storage vessels under anaerobic conditions until required for subsequent use. Maintenance of yeast quality during storage is critical for subsequent fermentation performance. An assumption is made in brewing that all strains benefit from storage at 3-4°C. To test this assumption a model working system was initially established to assess cooling times of lager yeast in different suspension media. Preliminary investigations focussing on freshly propagated yeast slurry demonstrated that whilst the deleterious effects of extremely high storage temperatures on lager brewing yeast physiology was in line with expectation, utilization of traditionally recommended storage temperatures does not necessarily benefit yeast physiology when compared to slurry maintenance at slightly higher temperatures. Genome-scale transcriptional analysis in slurries cropped following an initial fermentation suggested that lager yeast might experience cold stress during slurry maintenance at typically recommended storage temperatures. In contrast, maintenance of lager yeast at a slightly higher storage temperature, in this case 10°C, yielded no adverse impact on key indicators of brewing yeast physiological state or on subsequent fermentation profiles following repitching into fermentations. Whilst these observations were not made using full production scale, they do indicate that optimal storage may not be currently being deployed for brewing yeast at full scale.

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## ABBREVIATIONS

| | |
|---|---|
| % | Percentage |
| °C | Degrees centigrade |
| µg | microgram |
| µL | microlitre |
| µs | microsecond |
| bp | Base pairs |
| cm | centimetre |
| g | gram |
| hr | hour |
| Kg | kilogram |
| L | litre |
| m | meter |
| M | Molar concentration |
| m/v | Mass concentration |
| mg | milligram |
| min | minute |
| mL | millilitre |
| mm | millimetre |
| mM | Millimolar |
| nm | nanometer |
| rpm | Revolutions per minute |
| s.g. | Specific gravity |
| sec | second |
| v/v | Volume concentration |
| YPD | Yeast extract, peptone and D-glucose medium |
| RO | Reverse Osmosis |
| DO | Dissolved Oxygen |
| DNA | deoxyribose nucleic acid |
| RNA | ribose nucleic acid |
| CSR | Cold Shock Response |
| NFR | Near Freezing Response |
| GSR | General Stress Response |
| CS | Cold Stress |
| CSP | Cold Shock Protein |
| EAdR | Early Adaptive Response |
| SAcR | Sustained Acclimatory Response |
| GPI | Glycosylphosphatidylinositol |
| CMV | Citrate Methylene Violet |
| VDK | Vicinal Diketone |

# CHAPTER 1:INTRODUCTION

## 1.1 A Brief History of Brewing

Brewing is an ancient tradition which is widely regarded as mankind's oldest biotechnology (Bamforth, 1998, Kunze, 1999, Boulton and Quain, 2001). The history of beer can be traced back almost 5000 years, its earliest mention being in Mesopotamian cuneiform writings dating back to 2800 B.C., with numerous illustrations and tomb furnishings occurring in ancient Egypt (Kunze, 1999). It was regarded as a diet staple and was routinely brewed by women in the home in the ancient world. Beer production only evolved into an "industry" when the breweries of Christian monasteries commenced sales of surplus beer for a small fee (Kunze, 1999). Indeed, it was the Bavarian brewing monks who noticed that some yeasts tended to sink during fermentation, and that beers brewed with such yeasts were more stable, resistant to infection, clearer and could be easily stored in the cool environments of vaults and caves (Corran, 1975).

The Industrial Revolution in the $18^{th}$ and $19^{th}$ centuries led to the introduction of technology for large scale beer production. Rapid urbanization and an efficient freight transport allowed commercial brewers to provide for the large markets, thus fuelling the conception of the commercial brewing industry (Hornsey, 2003). Apart from domestic growth, widespread trade and colonisation meant that the brewing style typically associated with Europe was spread globally to new geographic locations such as the United States of America, Australia and even Japan (Kunze, 1999, Hornsey, 2003). After initial

dominance, top-fermented ale beers were superseded by lager beers in terms of global popularity and sales.

Mergers and acquisition towards the end of 20$^{th}$ century resulted in the formation of the large brewing companies that exist today, with huge global scales of production (Gourvish, 1994). This has necessitated the continuous introduction of newer technology for seamless control of the production process (Kunze, 1999). More recently, the growing environmental consciousness and cost pressures have compelled the identification of energy saving avenues across the brewing process so as to reduce the carbon footprint of the brewery whilst still maintaining product quality and brand identity. This necessitates research which in turn is challenging the long accepted practises of brewing using modern tools and techniques.

## 1.2 Modern Brewing Process

The four principal ingredients utilized for beer production are water, barley, hops and yeast. Other materials such as adjuncts may be used for improving product quality (different colours, better foams, diverse flavours) or reducing process costs (Bamforth, 2006). The basic outline governing the transformation of these constituents into beer consists of malting (Section 1.2.1), milling (Section 1.2.2), mashing (Section 1.2.3), wort boil (Section 1.2.4), fermentation (Section 1.2.5), maturation and conditioning (Section 1.2.6).

### 1.2.1 Malting

Malted barley is the principal cereal of choice for malting whilst others such as wheat, rye, sorghum, oats or millets are used to a lesser extent (Briggs *et al.*,

2004) . After cleaning the barley stock of fragments of straw, pieces of wood, stones, dust and broken kernels via different machines (usually sequentially connected) (Kunze, 1999), the process of malting is initiated by hydrating or "steeping" the grain in aerated water maintained at a specific temperature. This increases the grain's water content to 42 – 48% (Briggs *et al.*, 2004) thereby initiating seed germination and the early stages of formation of a barley embryo. The exposure of embryo to moisture prompts the formation of hydrolytic enzymes such as amylases and the hormone gibberelic acid which triggers the formation of starch degrading enzymes in the aleurone layer (Boulton and Quain, 2001, Briggs *et al.*, 2004). The collective action of these enzymes is required to catalyze the degradation of starch present in the endosperm into fermentable carbohydrates during the mashing process. The entire process also makes the grain softer thereby facilitating the milling process (Briggs *et al.*, 2004). Depending upon the grain's kernel size and overall quality, the rate of germination is tightly controlled by regulating steeping time, steeping temperature and oxygen availability (through periodic aeration) (Kunze, 1999).

Following appropriate growth and optimum enzyme production, the germination process is stopped by a process called kilning to avoid utilization of sugar for plant growth (Briggs *et al.*, 2004). Kilning involves heat-drying the green malt (green implying to immature malt) at a specific temperature using hot air. Whilst this results in cessation of embryo growth the starch hydrolysing enzymes are preserved for subsequent release of fermentable sugars (Lewis, 1995). Differing regimes of kilning temperature and duration give rise to a wide variety of malts which eventually determines beer colour

and flavour (Briggs *et al.*, 2004, Lewis, 1995). Indeed, the diversity in malt variety is a major factor that allows the production of a plethora of beer types all over the globe. Following kilning the malt is cooled along with removal of brittle roots and dust making it suitable for long term storage (Kunze, 1999).

### 1.2.2 Milling

In order to give the malt enzymes better access to the endosperm for subsequent degradation of malt starch during mashing, the malt corn must be broken into smaller fragments. This is achieved by a mechanical process called milling whereby dried malt is fragmented into a coarse flour termed as "grist" (Schwarz *et al.*, 2002, Briggs *et al.*, 2004). Care should be taken to generate optimal particle size and cause minimal husk disintegration during milling because husk act as a filter material during mash separation, especially when a lauter tun is used as the mash separation system (Kunze, 1999, Schwarz *et al.*, 2002).

### 1.2.3 Mashing

Mashing is the process of producing a fermentable extract, termed as wort, from ground barley. In this process, the grist is mixed with an optimal volume of hot water, termed as liquor in the brewing industry, in a mash conversion vessel or the mash tun and mixed to maintain homogeneity. The temperature of the liquor and subsequent mashing in mash conversion vessel is tightly controlled. This permits the optimization of the activity of the large number of malt enzymes acting simultaneously on grist components for maximum degradation of starch to release fermentable sugars, such as glucose, sucrose, fructose, maltose and maltotriose, and non-fermentable dextrins (Kunze, 1999, Lewis, 1995, Boulton and Quain, 2001). Adjuncts such as cereals, flours, or

other non-malted carbohydrates that supplement barley malt may then be added to extend the range of fermentable sugars (Briggs *et al.*, 2004).

Mashing temperatures and times have considerable impact on wort quality. Generally, higher mash temperatures favour rapid starch conversion whilst lower temperatures generate increased concentrations of total soluble nitrogen (Boulton and Quain, 2001, Briggs *et al.*, 2004). For lager beers, the mash temperature generally starts at low levels followed by recipe-dependent step wise increments for starch gelatinization (since solid starch granules are not readily degraded) and α-amylase activity to ensure progressive degradation of proteins and carbohydrates (Lewis, 1995, Briggs *et al.*, 2004). Final rise in temperatures to as high as 76-78 °C prevents further enzyme action and reduces the viscosity of the liquid phase for ease of filtration (Briggs *et al.*, 2004).

The "sweet wort" produced at the end of mashing is separated from the spent grains by lautering or mash filtration. Lautering is achieved via a lauter tun which has a false bottom with slots that retains grains whilst the wort drains away unhindered. Mash filters allow for more effective and rapid filtration although they might not result in production of bright wort (Briggs *et al.*, 2004).

### 1.2.4 Wort Boil

Following separation, the clarified sweet wort is transferred to a kettle also known as the copper (referring to the metal from which kettle was traditionally made). The wort is then boiled in the copper. This heat treatment permits inactivation of malt enzymes, sterilizes the wort from any contaminating

microorganisms, fixes its carbohydrate composition and concentrates wort gravity through water evaporation (Briggs *et al.*, 2004, Miedener, 1978, Bamforth, 1998). Wort boiling also causes protein coagulation and protein-polyphenol complex formation to generate a mixture called trub. Generally hops are added during wort boil, in the form of pellets or extract, either early in the boiling cycle or towards the end. Boiling causes extraction and isomerisation of hop α-acids into iso-α-acids which impart the characteristic bitterness to beer, besides formation of other flavour and colour complexes (Boulton and Quain, 2001, Briggs *et al.*, 2004).

After the boil is complete the solid trub is separated from hopped, sterile wort either in a hop-back (vessel with a slotted base) or a whirlpool (creates a vortex allowing solid settlement), cooled (using a heat-exchanger or paraflow) and pumped into a fermentation vessel (FV).

### 1.2.5 Fermentation

Yeast is the central character in fermentation and has also been regarded as the key player in brewing (Lodolo *et al.*, 2008). The primary goal of fermentation is the conversion of fermentable wort sugars into ethanol, carbon dioxide and secondary metabolites, including flavour compounds. Although FVs differ greatly in their shape and size, cylindroconical vessels are currently the dominant vessel type.

Following transfer, the cooled wort is maintained in the FV at a desired temperature depending upon the type of beer to be produced. Ale beers are generally fermented at temperatures ranging between 15 – 20 °C whilst lagers are formed by cooler fermentations at 7 – 15 °C. Currently, the majority of

breweries operate fermentation in batch mode with an initial dose of oxygen provided at the start of the fermentation process, mainly through direct oxygenation or air-saturation of the wort. Wort inoculation or "pitching" signals the beginning of fermentation and is usually performed at a specific rate, known as pitching rate. The molecular oxygen initially present in wort is rapidly utilized by yeast cells for synthesis of sterols and unsaturated fatty acids which are required for membrane formation, a pre-requisite for cell budding and yeast growth (David, 1973, Kirsop, 1991). As a result, batch brewing fermentations typically display an early, short aerobic environment followed by a prolonged anaerobic phase due to oxygen depletion and carbon dioxide accumulation. Apart from oxygen, simultaneous uptake of fermentable sugars and nitrogen compounds (both organic and inorganic) through permeases located in the cell membrane is crucial for cell growth since it acts as a source of carbon and energy. In the majority of yeasts carbon utilization occurs via glycolysis resulting in the conversion of glucose into pyruvate along with the synthesis of ATP. Being facultative anaerobes, brewing yeast can further the carbon flow via oxidative respiration (comprising of Krebs cycle and oxidative phosphorylation) or fermentation.

Whilst oxidative phosphorylation yields carbon dioxide and higher amounts of energy in comparison to the fermentative pathway, the lack of oxygen during the bulk of the fermentation period ensures dominance of the fermentative pathway, resulting in ethanol production. However, oxygen availability is not the only factor governing the switch between respiration/fermentation and other regulatory mechanisms (such as catabolite repression, the Crabtree- and Kluyver-effect) dependent upon strain genotype and sugar concentrations

affect the respiration/fermentation balance (Lagunas, 1986, Lagunas, 1979). Despite oxygen availability throughout early fermentation, high sugar concentrations subject brewing yeast to strong carbon catabolite repression leading to reduction or complete lack of activity of respiratory enzymes and decreased respiration capability (Lagunas, 1979, Lagunas, 1986). The secondary metabolites produced during fermentative metabolism impart both favourable and unfavourable flavour characteristics to the final product.

As nutrients deplete yeast proliferation ceases and fermentation slows down with the achievement of attenuation (completed conversion of wort sugars to ethanol by the yeast). Towards the end of the fermentation, yeast cells flocculate or aggregate into clumps and either sink to the bottom of the vessel as is the case for lager yeast (also termed bottom-fermenting yeast) or float to the top as is the case for ale yeast (also termed top-fermenting yeast). The cells are collected or "cropped" and stored for their re-utilization in a subsequent fermentation. This marks the end of primary fermentation and the beer thus produced (called as "green beer") is hazy, contains insufficient carbon dioxide with non-optimal taste and aroma (Lewis, 1995, Boulton and Quain, 2001, Briggs *et al.*, 2004). Hence the "green beer" requires further conditioning or maturation.

### 1.2.6 Maturation & Conditioning

Maturation of green beer can either be performed through a secondary fermentation or ageing. A secondary fermentation requires reduced yeast activity (achieved by minimal cell counts) and is usually performed at low temperatures in closed vessels (for lagers) or in cask (traditionally for ales).The

low temperature clarifies the beer by facilitating natural sedimentation of remaining proteins and polyphenol complexes (Bamforth, 1998). Yeast then assimilates unwanted flavour compounds, such as diacetyl, to below its flavour and aroma threshold concentrations and generates carbon dioxide which "conditions" the beer into its mature form (Briggs *et al.*, 2004). Yeast is not a pre-requisite for beer maturation and carbonation and flavour adjustment can also be performed by gas sparging and venting undesirable flavour metabolites into the atmosphere, collectively the process is termed maturation or "ageing" (Lewis, 1995, Boulton and Quain, 2001, Briggs *et al.*, 2004). Due to the instability of naturally conditioned beers for extended periods, the majority of breweries tend to favour ageing as the method of beer maturation (Bamforth, 2006).

Following conditioning, matured beer is chilled and filtered for removal of residual yeast and is then termed bright beer. This is transferred into bright beer tanks (BBTs), carbonated for achieving a specific carbon dioxide content and is ready for packaging into kegs, bottles, cans or other packaging forms (such as PET bottles) depending upon the point of sale. Before packaging, the beer may be sterile filtered or filled bottles/cans might be subjected to pasteurization (controlled heat treatment).

### 1.3 Brewing Yeast

A wide array of beer styles have developed over time by the industry, however, the utilization of brewing yeast for beer production has been a constant factor. Brewing yeast are unicellular, eukaryotic fungi (Barnett, 1979) belonging to the genus *Saccharomyces* and can generally be classified into two categories - ale and lager yeasts (names derived from their respective products). Ale and

lager yeast are also referred to as top- or bottom-fermenting because of their respective characteristic rise or sedimentation in the fermentation vessel. The former process relies on hydrophobic interaction of the yeast cell walls with carbon dioxide bubbles (Amory and Rouxhet, 1988), the latter on lectin-mediated cell to cell interactions leading to the formation of yeast clumps or flocs (Dengis *et al.*, 1995).

Ale and lager yeast possess distinct phenotypic characteristics which can be exploited for strain differentiation. These include differences in colony morphology, cell size (increased size in lager yeast due to ploidy) (Vagvolgyi *et al.*, 1988) and appearance (chain formation in ale yeast), growth at 37 °C (ale but not lager yeast) (Boulton and Quain, 2001), ability to utilize chromogenic substrate (melibiose utilization by lager but not ale strains) (Barnett, 1979), flocculation profiles, ability to assimilate maltose/maltotriose at low temperatures (lager yeast) (Zheng *et al.*, 1994) and flavour generation (enhanced sulphite production) (Crumplen, 1993).

Besides physiological characteristics, lager yeast (*Saccharomyces cerevisiae syn. S. pastorianus)* differ from ale yeast (*Saccharomyces cerevisiae)*, in its genetic composition. *S.pastorianus* is an interspecies hybrid (Martini and Kurtzman, 1985, Tamai *et al.*, 1998) comprising of two nuclear sub-genomes originating from *S.cerevisiae* and *S.bayanus* (Fujii *et al.*, 1996a, Kodama *et al.*, 2001) and a single mitochondrial genome arising from *S.bayanus* (Groth *et al.*, 2000, Nakao *et al.*, 2009). It consists of multiple copies of 16 different chromosomes, some of which are chimeric due to translocation events between the two nuclear sub-genomes (Nakao *et al.*, 2009, Smart, 2007). *S.pastorianus* is an allopolyploid and possesses multiple copies (1 – 6) of most genes

including those responsible for maltotriose uptake and sulphite production thereby conferring characteristic properties to lager yeast (Nakao *et al.*, 2009).

Different *S.pastorianus* isolates are known to contain different combinations of the genomes of four closely related species (*S. cerevisiae*, *S.bayanus*, *S.bayanus var uvarum* and a yet unclassified "lager type" species) which together comprise the *Saccharomyces sensu stricto* group (Casaregola *et al.*, 2001, Naumov *et al.*, 1993). Lager yeast, thus, have been divided into two groups (Dunn and Sherlock, 2008, Liti *et al.*, 2005). The ancestors for Group 1 (Saaz type) strains have been proposed to arise from a mating event between an ale-producing *S.cerevisiae* and *S.bayanus* followed by post-hybridisation loss of large portions of the parent *S.cerevisiae* sub-genome. The ancestors for Group 2 (Frohberg type) strains have been proposed to be separate but closely-related to the parent strains for Saaz type *S.pastorianus*, although in this instance loss of the parent *S.cerevisiae* genome was considerably less (Dunn and Sherlock, 2008). Higher retention of the genome of the cold-tolerant *S.bayanus* (Giudici *et al.*, 1998) in both groups is a result of selection pressure imposed by centuries of low temperature fermentations (Dunn and Sherlock, 2008).

## 1.4 Yeast Management in the Brewery

Yeast management in a brewery can be viewed as comprising of all the steps associated with managing yeast in the brewing process. Usually this comprises the processes of laboratory storage (Section 1.4.1), yeast propagation (Section 1.4.2), pitching (Section 1.4.3), fermentation (previously described in Section 1.2.5), cropping (Section 1.4.4), storage (Section 1.4.5) and acid washing (Section 1.4.6). Apart from laboratory storage, the remaining processes are

often repeated in the brewery in a cyclic manner and have been termed as "serial repitching". The manner in which yeast is handled in the brewery critically influences its physiological and genetic status (O'Connor-Cox, 1997, Boulton and Quain, 2001, Gibson *et al.*, 2007). Any inconsistencies can cause variability in yeast status resulting in aberrant fermentations and compromised beer quality (Heggart *et al.*, 1999, Boulton and Quain, 2001).

### 1.4.1 Laboratory Storage

The storage and assured supply of pure and stable yeast culture is important for efficient yeast management. For long term storage the method should be able to maintain cell viability, its genetic stability and ideally should be feasible for routine practise (Quain, 1995). Some of the routinely applied storage practises include sub-culturing on solid agar or liquid broth, dehydration to yield a powder form and culture freezing in liquid nitrogen (Kirsop, 1991). The methods, however, differ in maintenance of shelf life and require varying degree of technical resources (Boulton and Quain, 2001).

### 1.4.2 Yeast Propagation

The large quantities of yeast required for industrial-scale fermentations necessitate the growth of yeast in specially designed vessels prior to fermentation. Pure yeast cultures from the laboratory are inoculated into wort and encouraged to grow in increasing volumes of the media so that a required biomass amount can be achieved (Jones, 1997, Kennedy, 2000). Initial propagation is usually conducted at laboratory-scale in order to obtain an inoculum size adequate for the first stages of brewery propagation (Boulton and Quain, 2001, Jones, 1997). Whilst laboratory propagations are performed at high temperatures for achieving maximum growth rates, the temperature is

usually reduced during the final stages of propagation in the brewery to prevent temperature shock following inoculation in the fermenter (Boulton and Quain, 2001, Jones, 1997). Irrespective of the scale of propagation, the gaseous environment is always aerobic (achieved through sterile air or oxygen) to stimulate growth rate and synthesis of sterols and fatty acids, crucial for yeast replication in subsequent fermentations (David, 1973, Boulton and Quain, 2001, Verbelen *et al.*, 2009c).

Reutilization of yeast biomass from one fermentation to inoculate a further fermentation (serial repitching) is common practise in the brewery. Such recycling can cause genetic drift and gradual deterioration in yeast physiology (Smart and Whisker, 1996, Jenkins *et al.*, 2003, Gibson *et al.*, 2007, Jenkins *et al.*, 2009). As a result the number of recycling events can vary between modern breweries anywhere between 15-20 or 5-10 generations (Boulton and Quain, 2001) and is generally pre-determined depending upon the strain stability. Periodic introduction of freshly propagated yeast eliminates the inconsistencies in strain properties besides preventing other microbial contaminants such as petite variants of the production strain, wild yeast and bacteria; although the initial fermentations utilizing freshly propagated yeast can demonstrate irregularities such as extended lag phase due to lack of cell-cycle synchrony (Miller *et al.*, 2012).

### 1.4.3 Pitching

The process of wort inoculation with yeast is referred to as "pitching" in the brewing industry. Pitching is usually performed with a pre-determined number of viable cells (pitching rate) to achieve a desired suspended cell count at the start of fermentation. Occasionally pitching could be controlled by addition of

a defined weight of wet yeast, although this method is less precise and is usually limited to small-scale operations (Boulton and Quain, 2001). Pitching should be performed under sterile conditions to avoid contamination and drop in cell viability which can result in an array of irregularities in the final product including haze formation, increased metal ion content and reduced flavour stability (Casey *et al.*, 1983, Mochaba *et al.*, 1996).

Pitching rate is usually strain-dependent and is determined on the basis of several other factors such as wort composition, gravity, fermentation temperature and the physiological characteristics of the production strain at the time of inoculation (O'Connor-Cox, 1997, O'Connor-Cox, 1998, Boulton and Quain, 2001). Increments in pitching rates have been proposed as a convenient method for increasing fermentation rates without affecting yeast physiology, although variations in flavour profiles particularly diacetyl concentrations were observed (Verbelen *et al.*, 2009a).

### 1.4.4 Cropping

Cropping comprises of the removal of sedimented yeast fraction at the bottom (cone) of cylindroconical fermentation vessels following completion of fermentation. In fermentations with lager yeast, the majority of the cells flocculate and sink to the bottom of the cone. Standard brewing practise usually incorporates a "VDK stand" once attenuation is achieved (Bamforth, 1998, Boulton and Quain, 2001). This involves maintenance of green beer in the fermenter so that suspended yeast cells can reduce the off-flavour imparting vicinal diketone (VDK) compounds into less flavour active forms (acetoin) (Pickerell *et al.*, 1991, Briggs *et al.*, 2004). Thus yeast is usually cropped after desired VDK concentrations have been achieved. However, the

prevalence of stressful environmental conditions at the end of fermentation such as nutrient depletion, high ethanol and carbon dioxide concentration can impose considerable stress on yeast populations (Gibson *et al.*, 2007). This can lead to reduced cellular viability, vitality and diminished reserve carbohydrate content thus compromising cellular function during subsequent storage and fermentation (Quain *et al.*, 1981, O'Connor-Cox, 1998). As a result, the timing of cropping is crucial for balancing adequate VDK removal and minimizing deterioration in yeast physiology.

Yeast sediment in the cone is not necessarily homogenous and sub-populations might exist within the cone exhibiting inconsistent physiology (Deans, 1997, Powell *et al.*, 2004). The crop sediment may also display an age gradient due to premature settling of older/highly-flocculent cells leading to their enrichment near the bottom part of the cone and accumulation of young/weakly-flocculent cells in the top layers (Deans, 1997, Powell *et al.*, 2003). Pitching fermentations with such older or younger populations can lead to extended lag phase and delayed onset of cell growth respectively (Powell *et al.*, 2000). Serial cropping and repitching of discrete crop layers may result in the selection of distinct fermentation characteristics (Jenkins *et al.*, 2003, Powell *et al.*, 2003, Powell *et al.*, 2004). Thus, best practise measures recommend removal of the mid-part of the crop sediment for subsequent storage and fermentation inoculation (Boulton and Quain, 2001, Kennedy, 2000). The remaining fraction can be used as animal feed.

### 1.4.5 Storage of cropped yeast

Usually cropped yeast is stored before re-pitching in the brewery to allow for operational flexibility. This particular process in brewery yeast handling is the

main focus of this thesis and hence has been outlined in greater depth in later sections of the thesis (Chapters 4 and 6). An overview of the process has been presented here.

The primary aim for any storage regime is to maintain strain integrity without any contamination and allow minimal deterioration in the physiological state of cropped yeast (McCaig and Bendiak, 1985b, Boulton and Quain, 2001, Lodolo *et al.*, 2008). Storage is thus performed at low temperatures and recommended values range from 0 – 4 °C (Murray, 1984, McCaig and Bendiak, 1985b, Boulton and Quain, 2001) so as to limit cellular metabolism and minimize any physiological impairment. However, application of near-freezing temperatures can impose considerable cold stress on yeast populations (Gibson *et al.*, 2007) along with formation of intracellular ice crystals that can cause cell death (O'Connor-Cox, 1998). Following cropping, yeast can either be stored as (i) a slurry consisting of cropped yeast cells in unprocessed or diluted beer from the fermenter or (ii) in the form of a pressed cake (O'Connor-Cox, 1998, Boulton and Quain, 2001) (described further in Chapter 6).

Slurry maintenance occurs in a closed vessel (to avoid contamination) under atmospheric pressure. A typical yeast storage vessel (also called as yeast collection vessel) has been shown in Figure 1.1. Cooling of yeast slurries in storage tanks is achieved using cooling jackets whilst hygiene is maintained by incorporating a "Cleaning in Place" (CiP) regime and avoiding microbial contamination (through microbiological quality filter) (Boulton and Quain, 2001). Slurry attemperation is achieved by gentle or periodic agitation via the mechanical agitator (Figure 1.1) to avoid thermal gradients and hot spots of metabolic activity (McCaig and Bendiak, 1985a, Lewis, 1995). The gaseous

environment inside the storage vessel should be strictly anaerobic to avoid utilization of the glycogen reserves since these are vital for fuelling lipid formation and membrane generation in the early phases of subsequent fermentation (Quain and Tubb, 1982) (McCaig and Bendiak, 1985b) (Boulton and Quain, 2001). Duration for slurry storage should be minimal since yeast activity in the vessel is only diminished and not completely eliminated; a storage period of 48 - 72 hours is deemed best practice (Boulton and Quain, 2001, Heggart *et al.*, 1999).

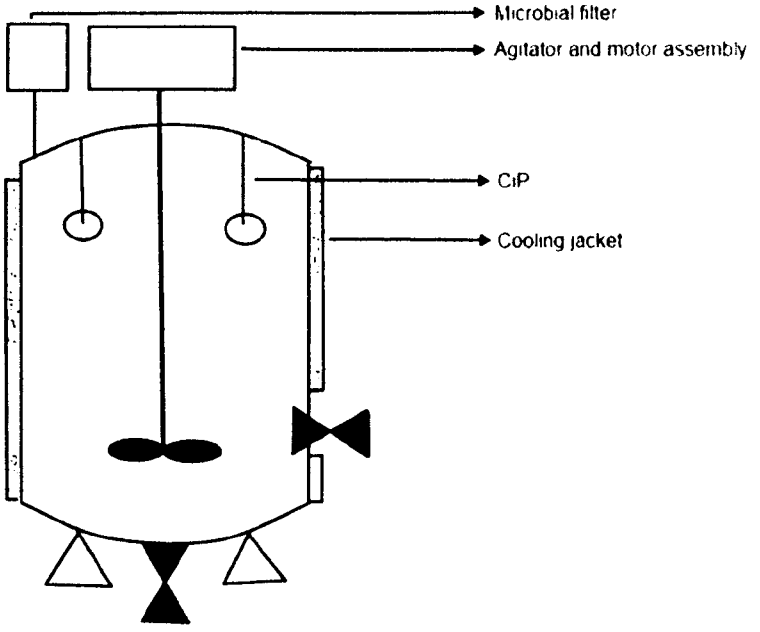



**Figure 1.1 – Schematic of a vessel for storage of cropped yeast slurry in the brewery. (Adapted from Boulton & Quain, 2001).**

### 1.4.6 Acid Washing

Despite best efforts, yeast handling in the brewery is generally prone to low levels of contamination from other microbes including bacteria, wild yeast and petite mutants. During active fermentation brewing yeast can outcompete most bacteria, although certain bacterial isolates can still flourish and can

compromise product quality and safety (Boulton and Quain, 2001). Contaminated pitching yeast can result in undesirable flavour characteristics and cloudy beer (Cunningham and Stewart, 1998). Thus, pitching yeast is typically treated with food grade acid in a process termed as acid washing (Boulton and Quain, 2001, Cunningham and Stewart, 1998, Simpson W. J., 1989). The low pH (2.2 – 2.5) is lethal for most common bacteria whilst does not cause much harm to brewing yeast (Boulton and Quain, 2001, Cunningham and Stewart, 1998); however acid washing is ineffective against wild yeast or petite contamination (Simpson W. J., 1989). Acid washing is typically performed at low temperatures (0 – 4 °C) for 2 - 4 hrs (Simpson W. J., 1989, Boulton and Quain, 2001) and the yeast slurry is well-mixed with either a particular or a mixture of mineral acids (such as phosphoric acid with ammonium persulphate), the latter having enhanced bactericidal activity probably due to the action of oxygen radicals associated with hydrogen peroxide formation (Simpson, 1987). Yeast resistance to acid washing is dependent upon its genetic and physiological status (Wilcocks and Smart, 1995) and the environmental conditions employed during acid washing. High temperatures and increased ethanol concentrations during acid washing can deleteriously effect yeast physiology presumably by compromising the yeast cell wall and membrane, thus leading to impaired cell viability and vitality (Fernandez, 1993, Simpson W. J., 1989).

## 1.5 Cold Stress during Yeast Handling in the Brewery

For unicellular organisms such as yeast, stress factors can generally be defined as those that lead to a reduction in growth rate (Ruis and Schuller, 1995). Yeast handling practices in the modern brewery can impose a plethora of stresses on

brewing yeast due to constant alterations in the environmental temperature, pH, osmolarity, nutrient availability along with suboptimal concentrations of oxygen (and derivatives), metal ions and ethanol (reviewed by Gibson et al 2007). This thesis focuses on those processes during yeast handling that involve yeast exposure to thermal downshift and thus have the potential to induce a cold stress response in brewing yeast.

Currently, lager fermentations constitute the vast majority of brewing fermentations worldwide. They are routinely conducted at temperatures between 7 – 15 °C which is much lower than the optimal growth temperatures for lager yeast (25 °C). *S.cerevisiae* typically demonstrates a cold-shock response at temperatures below 20 °C (Kondo and Inouye, 1991); lager yeast, however, is more cold-tolerant than *S.cerevisiae* due to the hybrid nature of its genome. In comparison to pitching with stored yeast slurry, low temperature fermentations utilizing freshly propagated slurry can cause more damage to brewing yeast physiology on account of the severity of the temperature drop. Cooling of the cylindroconical fermentation vessels towards the end of fermentation followed by thermal gradients in the cone routinely impose extreme temperature variations on brewing yeast. Passage of cropped slurry through a chiller (usually at 4 °C), slurry maintenance in storage tanks (at 2-4 °C) and cold acid wash treatment (at 4 °C) exposes yeast to thermal downshift. Thus, yeast handling comprises of a series of low temperature environments, often imposed in a cyclic manner due to serial-repitching, which can lead to brewing yeast experience cold stress.

## 1.6 Cold Stress

Most prokaryotes and eukaryotes are exposed to a number of environmental changes both in their natural habitat and during industrial applications. These can impair or reduce their growth rate and are deemed "stressful" to the microorganisms. Alterations in ambient environmental temperatures are commonly experienced by a large number of organisms, from bacteria to mammals. Exposure to high environmental temperatures or "heat shock" was one of the earliest recognized forms of environmental stresses in yeast which has led to our comprehensive understanding of cellular response to heat shock. On the other hand, the mode of cellular adaptation following exposure to a downshift in environmental temperature or "cold shock/stress" is relatively less well understood (Al-Fageeh and Smales, 2006).

A reduction in ambient temperature has been demonstrated to elicit a transcriptional or translational response in many organisms including bacteria such as *Escherichia coli* (Jones *et al.*, 1987) and *Bacillus subtilis* (Lottering and Streips, 1995), yeast (Kondo and Inouye, 1991), plants (Salerno and Pontis, 1989) and animals (Tiku *et al.*, 1996). However, different kingdoms of organisms seem to have different responses and a conserved set of cold inducible genes or proteins has not yet been identified (Thieringer *et al.*, 1998). In bacteria, a downshift in temperature induces the production of a specific class of proteins termed as the cold shock proteins (CSPs) and is accompanied by repression of global transcription and translation. Although most CSPs are pivotal for cell survival, their actions are extremely inter-dependent highlighting a cooperative response (Jones *et al.*, 1987, Jones and Inouye, 1994, Thieringer *et al.*, 1998). Following initial adaptation, the synthesis of

CSPs is diminished and global protein synthesis resumes. The bacterial CSPs are highly conserved and almost all exhibit single-strand nucleic acid binding activity (Jones and Inouye, 1994, Thieringer *et al.*, 1998).

### 1.6.1 Physical effects of Thermal Downshift

Thermal downshift has significant impact on the functioning of the yeast cell membrane. This, in turn, is responsible for most of the deleterious effects of cold stress on cell phenotype. Within the yeast cell membrane, the phospholipid moieties are arranged in a bilayer with polar heads interacting with the intra- and extra-cellular aqueous phases and the fatty acyl chains are stacked in parallel at right angles to the plane of the membrane. Normally the yeast cell membrane is flexible and present in a liquid crystalline form (Thieringer *et al.*, 1998). Thermal downshift leads to increased membrane viscosity and at a typical temperature the membrane undergoes a phase change to a gel phase (Quinn, 1988, Sinensky, 1974). This reduces membrane fluidity and impairs cell growth by hindering the function of membrane bound enzymes, slowing down diffusion rates and affecting cross membrane protein /metabolite transport (Mager and Ferreira, 1993, Ruis and Schuller, 1995, Al-Fageeh and Smales, 2006).

Yeast overcomes this increase in membrane rigidity by a phenomenon known as homeoviscous adaptation (Sinensky, 1974). This most commonly involves unsaturation of the phospholipid fatty acid chains which increases disturbance in the membrane and enhances membrane fluidity (Beales, 2004). Other alternative mechanisms such as reduction in the mean fatty acid chain length and an increase in mono-unsaturated straight chain fatty acids can also enhance

membrane fluidity by causing fewer carbon – carbon interactions between neighbouring chains and increasing membrane entropy (Beales, 2004, Beney and Gervais, 2001). These adaptations can restore normality in membrane function by resuming solute transport and normal functioning of membrane bound enzymes.

### 1.6.2 Effect of Thermal Downshift on Yeast Gene Expression

Yeast adaptation to a change in environmental temperature involves considerable regulation in its global gene expression (Lashkari *et al.*, 1997, Sahara *et al.*, 2002, Schade *et al.*, 2004, Murata *et al.*, 2006). Studies investigating the cold-stress response in *S. cerevisiae* have reported conflicting results (Lashkari *et al.*, 1997, Gasch *et al.*, 2000, Sahara *et al.*, 2002, Kandror *et al.*, 2004, Murata *et al.*, 2006), which can be attributed to subtle variations in experimental parameters. However, there are certain common elements in the yeast cold response.

Low temperatures cause stabilization of the nucleic acid secondary structures (Thieringer *et al.*, 1998) and hinder both polymerase and ribosomal functioning (Mager and Ferreira, 1993). As a result, a downshift in temperature results in decreased transcriptional and translational efficiency (Mager and Ferreira, 1993, Thieringer *et al.*, 1998, Phadtare *et al.*, 1999). Indeed, investigators have reported a reduction in the expression of majority of genes except for certain genes whose induction helps the cell to cope up with the stressful environment (Sahara *et al.*, 2002, Schade *et al.*, 2004).

Primarily, the genetic response seems to be dependent upon both the severity of low temperature (temperature-specific) and the duration of exposure (time-specific) (Sahara *et al.*, 2002, Schade *et al.*, 2004, Murata *et al.*, 2006, Aguilera

*et al.*, 2007). It has been proposed that yeast elicits different responses to temperature drops in ranges of 10-18°C and below 10°C (Al-Fageeh and Smales, 2006). These responses have been termed as the cold shock response (CSR) and near-freezing response (NFR) respectively (Al-Fageeh and Smales, 2006) Perhaps, the most crucial point of distinction between the two responses is the lack of any active growth by *S.cerevisiae* in the near freezing response as opposed to the temperature range of 10-18°C where after a certain period of acclimatisation yeast can grow actively (Gasch *et al.*, 2000).

### 1.6.3 Cold Shock Response (CSR)

In *S. cerevisiae*, the cold shock response (CSR) (10 – 18 °C) can be delineated into three distinct phases - early, mid and late phase (Sahara *et al.*, 2002). The early/mid phase is characterized by the initial up-regulation of genes associated with the transcriptional followed by the translational machinery (Al-Fageeh and Smales, 2006). At 10°C, *S.cerevisiae* appears to compensate for the reduction in translational efficiency by de novo ribosome synthesis. The mid-phase also involves regulation of genes associated with cytosolic ribosomal proteins (Al-Fageeh and Smales, 2006). This is followed by the activation of the general stress response in late phase (Al-Fageeh and Smales, 2006, Schade *et al.*, 2004). Thus the CSR has been defined as an extremely coordinated reaction involving the regulation of 'sequential waves' of genes encoding different modules of cellular machinery (Al-Fageeh and Smales, 2006).

### 1.6.4 Near Freezing Response (NFR)

As mentioned earlier, complete lack of cell growth has been proposed to occur at near-freezing temperatures. The near-freezing response

(NFR) in *S.cerevisiae* involves dramatic induction (up to 7-fold) of the trehalose synthesizing genes along with increased expression of certain heat shock proteins encoded by *HSP12, HSP42, HSP104* and *SSA4.* This suggests partial (if not complete) activation of the general stress response (Aguilera *et al.*, 2007, Murata *et al.*, 2006). The NFR is also characterized by up-regulation of other factors responsible for facilitating cellular transition back to optimal temperatures. This includes the neutral trehalase encoding NTH1p (associated with trehalose hydrolysis during normal growth) (Nakagawa *et al.*, 2002) and chaperonin CCT (associated with proper folding of tubulin and actin proteins) (Somer *et al.*, 2002).

### 1.6.5 General (Environmental) Stress Response (GSR)

The GSR is regarded as an evolutionary adaptation enabling yeast cells to respond to unfavourable environmental conditions in a non-specific manner so as to maintain cellular fecundity whilst stress-specific responses are being activated (MartinezPastor *et al.*, 1996, Gibson *et al.*, 2007). Stimulation of the GSR has been observed following exposure to both natural and industrial borne stresses, including brewing fermentations (Gibson *et al.*, 2008). The GSR involves cooperative regulation of around 200 genes encoding metabolic proteins (glycogen and trehalose), heat-shock proteins and transcription factors. GSR-mediated gene regulation occurs via the activation of the zinc-finger transcriptional activators Msn2p and Msn4p which bind to the stress responsive element (STRE). (Smart, 2007, Gasch *et al.*, 2000, Marchler *et al.*, 1993). *MSN2/MSN4* activation has also been proposed to account for yeast acquiring resistance to one type of stress following exposure to a different, completely unrelated form of stress (Lindquist, 1986, Thieringer *et al.*, 1998).

Reversal to optimal environmental conditions prompts rapid degradation of Msn2p thus highlighting the GSR's transient nature of (Bose *et al.*, 2005).

### 1.6.6 Cold - Responsive Genes in Yeast

As mentioned earlier, up-regulation of a number of specific genes has been associated with yeast adaptation and its recovery from cold shock. The most prominent ones have been introduced for the purpose of this thesis.

#### 1.6.6.1 TIR/DAN and Seripauperin Gene Families

The *TIR/DAN* gene family primarily consists of 8 members which largely encode for putative mannoproteins attached to the cell wall polysaccharides via covalent bonds (Sertil *et al.*, 1997, Abramova *et al.*, 2001a, Abramova *et al.*, 2001b). Most proteins encoded by the *TIR/DAN* genes have an endoplasmic reticulum localization sequence (at the N-terminal), a central serine/threonine rich central domain and a hydrophobic C terminus containing a signal for glycosylphosphatidylinositol (GPI) anchorage (Abramova *et al.*, 2001a, Abramova *et al.*, 2001b). Using northern hybridization, Kondo and Inouye (1991) initially demonstrated that *TIP1* mRNA was strongly induced by both cold- and heat-shock in *Saccharomyces cerevisiae.* The induction of *TIP1*, *TIR1*, *TIR2* and *TIR4* following exposure to or growth at low temperatures (both 10 and 4 °C) has now been widely recognised (Murata *et al.*, 2006, Homma *et al.*, 2003, Schade *et al.*, 2004, Panadero *et al.*, 2006, Sahara *et al.*, 2002).

Possessing close amino acid-sequence resemblance to the TIR/DAN genes is the seripauperin or PAU gene family, the largest gene family in *S.cerevisiae* (Luo and van Vuuren, 2008). It shares 85-100 % gene identity and a conserved

PAU domain with the N-terminal region of TIR/DAN proteins (Abramova *et al.*, 2001a), although PAU proteins have a poorer serine content (hence the name seripauperins) (Viswanathan *et al.*, 1994). Like the *DAN/TIR* genes, a subset of the *PAU* genes (*PAU1, PAU2, PAU4, PAU5, PAU6* and *PAU7*) have also been demonstrated to be up-regulated following exposure and extended incubation at 4 °C (Homma *et al.*, 2003, Murata *et al.*, 2006).

Apart from acting as characteristic cold-stress biomarkers, induction of *TIP1*, *TIR1* and *TIR2* (along with *DAN1*) (Cohen *et al.*, 2001) has also been observed during anaerobiosis (Donzeau *et al.*, 1996, Abramova *et al.*, 2001a). Under anaerobic conditions, induction of the *TIR/DAN* gene subset is achieved by the transcriptional factor encoded by *UPC2/MOX4*, through the *AR1* consensus element (Cohen *et al.*, 2001). A lack of induction of the *TIR* genes was observed in a mox4 deletion mutant (Abramova *et al.*, 2001a) following temperature downshift. It has thus been suggested that just like under anaerobiosis, *MOX4* might act as the transcriptional activator for *TIR* genes following cold shock.

### 1.6.6.2 NSR1 and other Ribosomal Protein Genes

*NSR1* is a widely recognised cold-responsive genes in *Saccharomyces cerevisiae* (Lashkari *et al.*, 1997, Sahara *et al.*, 2002, Schade *et al.*, 2004, Murata *et al.*, 2006). The *NSR1* protein (NSR1p) is structurally related to mammalian nucleolin and is involved in pre-rRNA processing and yeast ribosome biosynthesis (Kondo and Inouye, 1991). Nsr1p is postulated to be an auxiliary factor for ribosome synthesis i.e. the gene is not absolutely essential for ribosomal synthesis (Kondo and Inouye, 1991). However, temperature downshift resulted in severely impaired pre-rRNA processing and reduced

growth in *NSR1* deletion mutants (Kondo *et al*, 1992). Cold-induced *NSR1* induction is reportedly maintained for extended periods (Sahara *et al.*, 2002, Schade *et al.*, 2004, Tai *et al.*, 2007). *NSR1* expression is not up-regulated by heat shock (Kondo and Inouye, 1991), although its regulation under other environmental stresses has not been widely investigated. Other cold responsive genes associated with the RNA machinery includes the helicase encoding *DED1*, *DBP2* and a vast number of ribosomal protein genes

### 1.6.6.3 Trehalose and TPS Gene Family

Accumulation of large amounts of the non-reducing disaccharide trehalose is important for cell viability maintenance following thermal downshift and has been deemed critical for cell survival at freezing temperatures (Kandror *et al.*, 2004). Apart from acting as a storage carbohydrate, trehalose serves as a stress protectant against a plethora of stresses (as part of the yeast GSR) and provides stability to protein structures and lipid membranes (Kwon *et al.*, 2003, Aguilera *et al.*, 2007, Gasch *et al.*, 2000). In yeast, the synthesis of trehalose is catalyzed by the Tps1/Tps2 complex which is composed of four different subunits encoded by the genes *TPS1*, *TPS2*, *TPS3* and *TSL1* (Kandror *et al.*, 2004, Kwon *et al.*, 2003). Tps1p and Tps2p form the catalytic domain whilst Tps3p and Tsl1p may act in the regulation or structural stabilization of the Tps1/Tps2 complex (Kwon *et al.*, 2003).

Marked induction (upto 20 fold) in *TPS1* and *TPS2* mRNA occurs in both the CSR and the NFR. This is due to the genes' enhanced transcriptional activation and the increased stability of its mRNA (Kandror *et al.*, 2004) at lower temperatures. Other components of the trehalose synthase complex, *TPS3* and *TSL1*, are induced in the near-freezing temperature range (Kandror *et al.*,

2004) and the late phase of cold shock at 10°C (Kandror *et al.*, 2004, Sahara *et al.*, 2002). Following return to non-stressful conditions, trehalose levels are lowered rapidly due to the activation of the trehalase encoding *NTH1*. Indeed, such non-requirement of trehalose under optimal growth conditions is the basis on which this disaccharide has been proposed as an indicator of the occurrence of stress in brewing yeast (Majara *et al.*, 1996).

#### 1.6.6.4 *OLE1* and *MGA2*

The gene *OLE1* codes for the only fatty acid desaturase found in *S.cerevisiae* (Stukey *et al.*, 1990, Nakagawa *et al.*, 2002) and catalyzes the formation of a double bond between carbons 9 and 10 of palmitoyl-CoA (16:0) and stearoyl-CoA (18:0), forming palmitoleic acid (16:1) and oleic acid (18:1) respectively (Zhang *et al.*, 1999). Thus, *OLE1* mediated unsaturation of the cell membrane is critical for achieving optimal membrane fluidity and curvature (Zhang *et al.*, 1999). Irrespective of the severity of thermal downshift, cold-mediated *OLE1* induction is transient with increased transcription immediately observed. following thermal downshift (Nakagawa *et al.*, 2002, Sahara *et al.*, 2002, Schade *et al.*, 2004). Stability of OLE1p has been deemed as an important factor for yeast growth at low temperatures (Loertscher *et al.*, 2006), although overexpression of the gene did not provide any added growth benefits (Kajiwara *et al.*, 2000). Apart from cold stress, expression of *OLE1* is regulated by nutrient fatty acid content in the growth media (Choi *et al.*, 1996) and remains unregulated under anaerobic conditions (Lai *et al.*, 2005) resulting in a lack of UFA formation in the absence of oxygen (Lorenz and Parks, 1991).

Nakagawa et al (2002) demonstrated that under conditions of cold stress *OLE1* transcription was under the control of the transcriptional factor encoded by

*MGA2*, contrary to earlier suggestion (Zhang *et al.*, 1999). MGA2p has been proposed to act as a putative sensor for low temperature (and hypoxia) (Nakagawa *et al.*, 2002) and regulates *OLE1* transcription via the ubiquitin/proteasome-dependent processing (RUP) pathway (Nakagawa *et al.*, 2002, Hoppe *et al.*, 2000). Following an external stimuli such as thermal downshift, the alteration of the lipid bilayer causes deubiquitination of the dormant, membrane attached MGA2p precursor leading to the release of an N-terminal fragment. This acts as a transcriptional activator of OLE1 by binding to the LORE (low oxygen responsive element) consensus sequence located in the gene's promoter site (Hoppe *et al.*, 2000, Aguilera *et al.*, 2007). Interestingly, other cold-shock and hypoxia inducible genes, including *TIP1*, also have the LORE sequence (Nakagawa *et al.*, 2002) suggesting that the LORE-binding complex might regulate the expression of a subset of cold-responsive genes (Vasconcelles *et al.*, 2001, Aguilera *et al.*, 2007).

#### 1.6.6.5 LOT Gene Family

Zhang *et al* (2001) reported the presence of five low-temperature-inducible (LOT) genes in *S. cerevisiae*; *LOT1/FBA1, LOT2/RPL2B, LOT3/NOP1* which encode proteins involved in glycolysis (Hammond *et al.*, 2005, Lorentzen *et al.*, 2004) ribosome biogenesis (Wojda *et al.*, 2003, Hammond *et al.*, 2005) and pre-RNA processing (Ren *et al.*, 2008, O'Connor-Cox, 1993) respectively. The functions of *LOT5* and *LOT6* remain unknown. *LOT1/FBA1* and *LOT3/NOP1* are essential genes in *S.cerevisiae* and disruption mutants for *LOT2/RPL2B* showed severely retarded growth phenotype at 10°C (Zhang *et al.*, 2001). Yeast exposure to near-freezing temperatures resulted in induction of all the *LOT* genes, except *LOT6* which demonstrated enhanced transcript

levels following thermal downshift to slightly higher temperatures, thus suggesting the involvement of diverse mechanisms for gene regulation.

## 1.7 Aims and Objectives

Yeast handling in the brewery routinely involves storage of cropped yeast biomass for repitching into a subsequent fermentation. The storage process usually occurs in a close stainless steel tank and the yeast is maintained under anaerobic conditions at temperatures ranging between 2 - 4 °C. However, the effects of brewing yeast storage on yeast quality and potential to perform in subsequent fermentation has not been systematically characterized. Moreover, recent investigations suggest that yeast exposure to near-freezing temperatures (such as 4°C or lower) can impose substantial cold stress on the microorganism (Leclaire and Smart, unpublished data) and may in fact be deleterious to yeast physiological state. The objective of the current work is to establish whether the temperatures routinely applied to cropped slurries during storage are optimal. To address this question the following approaches were applied:

(1) an investigation into the impact of storage temperature on key yeast physiological indicators typically deployed by the industry was undertaken;

(2) an analysis of the transcriptome was conducted to establish the impact of storage temperature on the expression of cold and general stress genes as well as genes key for the potential to perform in subsequent fermentation;

(3) an assessment of the impact of storage temperature on performance during subsequent fermentation.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 Yeast Strains & Storage

The yeast strains used in this study were: *Saccharomyces cerevisiae* (*Syn Saccharomyces pastorianus*) lager strain *W34/70*, haploid yeast *Saccharomyces cerevisiae* laboratory strain *S288C*, lager yeast NCYC 1116 and *Saccharomyces cerevisiae* ale yeast NCYC 2593. The sources of all strains used are outlined in Table 2-1. Stock cultures were cryopreserved in glycerol (20% v/v) supplemented YPD (1% w/v yeast extract, 2% w/v neutralized bacteriological peptone and 2% w/v D-glucose) at -80 °C whilst working cultures were maintained on standard YPD agar slopes containing 1% (w/v) yeast extract, 2% (w/v) neutralized bacteriological peptone, 2% (w/v) glucose and 1.2% (w/v) technical agar at 4°C. All above mentioned media were obtained from Thermo Fisher Scientific (UK).

| Yeast Strain | Type | Source |
|---|---|---|
| *Saccharomyces* W34/70 | lager | Hefebank Weihenstephan GmbH (Germany) |
| NCYC 1116 | ale | National Collection for Yeast Cultures (UK) |
| NCYC 2593 | ale | National Collection for Yeast Cultures (UK) |
| S288C | Lab Strain | National Collection for Yeast Cultures (UK) |

**Table 2-1 – Yeast strains used in the current study.**

### 2.1.1 Slope formation and maintenance

Yeast strains were propagated from cryopreserved cultures maintained at -80 °C. A small aliquot (50 µL) of the stock cryopreserved culture was step-wise propagated in 10 mL and 250 mL of YPD at 25°C for 3 days each. 1 mL aliquots of the "propagating culture" was inoculated onto several YPD agar

slopes and maintained as stock culture at 4 °C for use in subsequent experiments.

## 2.2 Growth Media

All experiments in this study were conducted either using YPD or industrial wort. YPD was prepared by dissolving 1% (w/v) yeast extract, 2% (w/v) neutralized bacteriological peptone and 2% (w/v) D-glucose in reverse osmosis (RO) water. YPD's constituent chemicals were acquired from Thermo Fisher Scientific (UK). All media were autoclaved at 121 °C at 15 psi for 15 min immediately after preparation. Experimental wort (hopped, sterile) with a specific gravity of 14 Plato was supplied by a commercial brewery immediately after boil. Care was taken to utilize the same batch of wort for each set of experiments to avoid media-inconsistency. Every batch of wort was analysed for its total extract content along with constituent concentrations of fermentable sugars and free amino nitrogen.

## 2.3 Differentiation between Ale and Lager yeast

### 2.3.1 Differential Yeast Growth Temperature

Using a sterile loop, representative yeast colonies were recovered from YPD slopes, streaked onto YPD agar (1% w/v yeast extract, 2%w/v neutralized bacteriological peptone, 2% w/v D-glucose, 2% w/v Agar) plates and incubated at 25, 34 and 37°C for 5 days. By dividing the agar plates into multiple quadrants, different experimental strains were streaked on the same plate to ensure identical temperature treatment and minimal experimental variation. Each strain was analysed in triplicate.

### 2.3.2 Differential Yeast Metabolism

#### 2.3.2.1 X-α-gal Test

Yeast colonies recovered from YPD agar slopes (using a sterile loop) were inoculated into 10 mL YPD broth and incubated at 27°C for 3 days under static conditions. Following culture harvesting (3000 rpm, room temperature, RT), the supernatant was discarded and yeast resuspended in 5 mL of sterile distilled water. 100 µl aliquots of each of the resultant cell suspensions were added to four wells (3 test and a control) of a 96 well microtiter plate. 10 µl of filter sterilized X-α-gal (5-bromo-4-chloro-3-indoyl-α-D-galactosidase) solution (6.25 mg / mL) prepared in 75% 1,2-propanediol (v/v in RO water) was added to each of the test wells whilst the control well comprised only RO water. Following 60 minutes incubation at 27 °C, wells were visually scrutinised for any colour change.

#### 2.3.2.2 Melibiose Test

Fermentation broth (0.45% w/v yeast extract, 0.75% w/v peptone) was prepared in RO water and dispensed in 2 mL aliquots in glass bijous containing small inverted Durham tubes. A pH indicator, bromothymol blue (0.005% w/v), was added to the broth followed by sterilization of the assembled apparatus. Filter-sterilized, 1 mL aliquots of melibiose [α-D-galactose-(1-6)-α-D-glucose] at a final concentration of 12% (w/v) were added to the sterile fermentation broth in test reactions whilst glucose (6% w/v) and RO water were respectively added in positive and negative controls.

Following yeast propagation in 10 mL YPD at 27°C for 48 hours, the cell suspension was harvested by centrifugation (3000 rpm, 10 minutes, RT). Resultant yeast pellet was washed once (RO water) and finally re-suspended in

10 mL sterile RO water. 100 µl of the washed propagating culture was inoculated into each of the melibiose fermentation broths and incubated at 27°C for 7 days. For each strain the analyses were performed in triplicates and the Durham tubes were monitored daily.

### 2.3.3 Extraction of total DNA (genomic and mitochondrial)

Representative yeast colonies from YPD agar slopes were inoculated into 100 mL YPD broth and incubated aerobically at 27°C for 3 days under continuous aeration. The cells were centrifuged at 3500 rpm for 10 minutes (RT) and utilized for total DNA (genomic and mitochondrial) extraction. Unless stated, all chemicals were obtained from Thermo Fisher Scientific (UK).

The cell pellet (0.2 g, wet weight) obtained, was washed in 5 mL of washing buffer (1M sorbitol, 50mM potassium di-hydrogen phosphate) and harvested again by centrifugation (3500 rpm, 10 minutes). Two volumes of washing buffer were added to the resultant cell pellet (assuming 1g wet weight of cells is equivalent to 1 mL volume). An aliquot (0.5 mL) of the resulting cell suspension, was added to equal volumes of sphaeroplasting buffer (1M Sorbitol, 50mM potassium di-hydrogen phosphate, 0.8% β-mercaptoethanol v/v) in a microcentrifuge tube (Eppendorf, Fisher Scientific, UK). 40µl of lyticase solution (200 units) was added and cell suspension incubated at room temperature for 45 minutes. The sphaeroplasts obtained, were harvested by centrifugation (13000 x g for 1 minute) and re-suspended in 1 mL of 50mM EDTA (pH 8.5). Sodium Dodecyl Sulphate or SDS (40 µL of a 10% w/v solution) was added and eppendorf tubes incubated at 65°C for 30 minutes. The mixture was cooled on ice for 5 minutes before addition of 200 µL of high

salt solution (3M potassium acetate, 2M glacial acetic acid) and further incubated at 65°C for 30 minutes. The lysate was centrifuged at 13000 x g for 30 minutes. The top 800µl of the supernatant was transferred to a fresh 1.5 mL eppendorf tube containing 600µl of iso-propanol and incubated for 5-10 minutes. DNA pellet was collected by centrifugation (13000 x g for 5 minutes) and washed once with 70% ethanol (v/v). The DNA pellet was then dissolved in 200 µL sterile water and treated with 20 µg of RNAse for 90 minutes at 37°C. DNA was extracted with phenol:chloroform (1:1, Sigma) followed by a further extraction with chloroform alone. The resulting DNA was precipitated using an appropriate volume (equivalent to $1/10^{th}$ of the total mixture volume) of 3M sodium acetate (pH 5.2) and 3 volumes of 100% ethanol. The precipitated DNA was collected by centrifugation and washed with ethanol solution (70%, v/v). Following centrifugation the supernatant was discarded and the DNA pellet was dissolved in 30 µL of PCR-grade water with subsequent storage at -20 °C. Quantification and purity of the isolated DNA was performed using the NanoDrop® (ND-1000, Thermo Scientific).

### 2.3.4 Polymerase Chain Reaction (PCR)

*W34/70* genomic DNA, extracted and quantified, as described in Section 2.3.3 was diluted using sterile PCR grade water (New England Biolabs, UK) to 240-250 ng / µL for subsequent amplification reactions. PCR reaction mixes were prepared as shown in Table 2-2. The PCR amplification conditions have been shown in Table 2-3. Details of the primer sequences used cannot be given as they subject to a patent application submitted by our laboratory.

| Components | Source | Single Reaction | |
|---|---|---|---|
| | | Volume (µl) | Concentration |
| Phusion HF Buffer | NEB (UK) | 4 | 1X |
| dNTPs | | 0.5 | 5 µM |
| FP | Eurofins | 0.5 | 50 pM |
| RP | Eurofins | 0.5 | 50 pM |
| Phusion DNA polymerase | NEB (UK) | 0.6 | 1.2 units |
| PCR grade water | Thermo Fisher | 10.9 | N/A |
| DNA | Self-prepared | 3 | 240-250 ng/ul |
| Total | N/A | 20 | N/A |

**Table 2-2 - PCR reaction mixture preparation for differentiation between lager and ale strains. FP and RP represent forward and reverse primer respectively. Source indicates the place of procurement of the corresponding components. Chemicals were added in the order listed.**

| Step | | Temperature | Time | Number of Cycles |
|---|---|---|---|---|
| Initial Denaturation | | 98 °C | 30sec | 1 |
| | Denature | 98 °C | 10sec | |
| Amplification | Anneal | 59 °C | 30sec | 35 |
| | Extension | 72 °C | 30sec | |
| Final Extension | | 72 °C | 5min | 1 |

**Table 2-3 - Thermal cycling parameters for PCR differentiation of ale and lager strains.**

## 2.4 Fermentation Vessels

### 2.4.1 Mini Fermentation vessels 1

Thermotolerance assessment of W34/70 was performed in miniature fermentation vessels (mFV) coupled with thermometers. A typical mFV-Thermometer system consisted of a sterile Wheaton glass serum bottle (Sigma-Aldrich, UK) with a pierced rubber septum at the neck through which a sterile alcohol thermometer was inserted for monitoring the media's temperature (Figure 2.1).

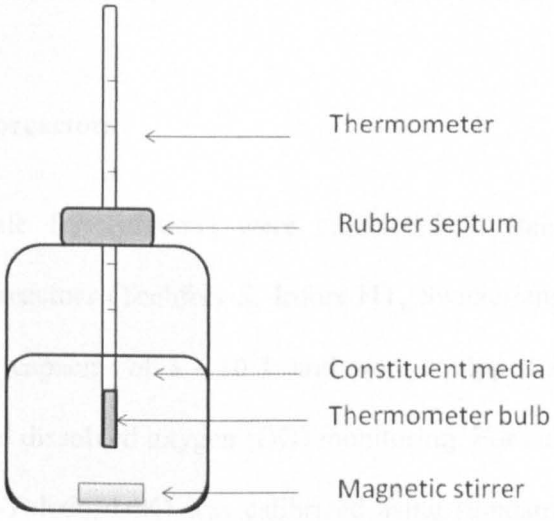



**Figure 2.1- Schematic of a mini fermentation vessel (mFV) coupled to a thermometer.**

### 2.4.2 Mini Fermentation vessels 2

Small-scale fermentations were conducted in sterile mFVs with a maximum capacity of 100 mL (Quain, 1985). Following inoculation, the mFVs were sealed tightly with a rubber septum reinforced by a metal crimp (Figure 2.2). Release of gas from within the mFVs was achieved through a Bunsen valve attached to a needle pushed through the septum (Figure 2.2). Magnetic stirrer at the bottom was utilized for agitation.

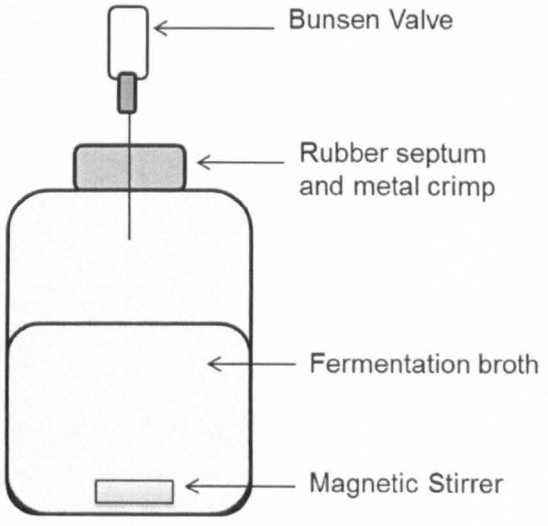



**Figure 2.2 - Schematic representation of a typical mini fermentation vessel (mFV).**

### 2.4.3 10L Bioreactors

Large-scale fermentations were conducted in stainless steel, in-situ sterilizable bioreactors (Techfors-S, Infors HT, Switzerland). The bioreactors had a working capacity of 3 - 10 L and were equipped for continuous pH, temperature and dissolved oxygen (DO) monitoring. For each bioreactor, a pH probe (Mettler-Toledo, U.K) was calibrated using standards at pH7 and pH4 before installation into an ingold port (25 mm) at the base of the vessel (see Figure 2.3). Subsequently, RO water (5L) was poured into the vessels. A Pt-100 temperature sensor (Techfors-S, Infors HT, Switzerland) was inserted through a pocket in the top plate until metal to metal contact could be heard (Figure 2.3). An electrode (TruDO, Finesse, Switzerland) for measuring dissolved oxygen (DO) was introduced through a separate ingold port (Figure 2.3) and was subjected to continuous RO water stirring (400 rpm) for at least 3hrs before sterilization to allow for polarization of the electrode's membrane. DO electrode calibration was performed in situ post sterilization. Sterilization

of the vessels was achieved at 121 °C (for 15 mins) by direct steam injection into the vessel jackets. The vessels were then cooled to 15 °C by cold water circulation through the vessel jackets. DO electrode was initially calibrated to 0% by flushing nitrogen into the vessel to replace water's oxygen content followed by 100% calibration by saturating the medium with air (at 15 L / min). After completion of the sterilization and calibration routine, the sterilization water was decanted through the bottom (sampling) port (Figure 2.3).

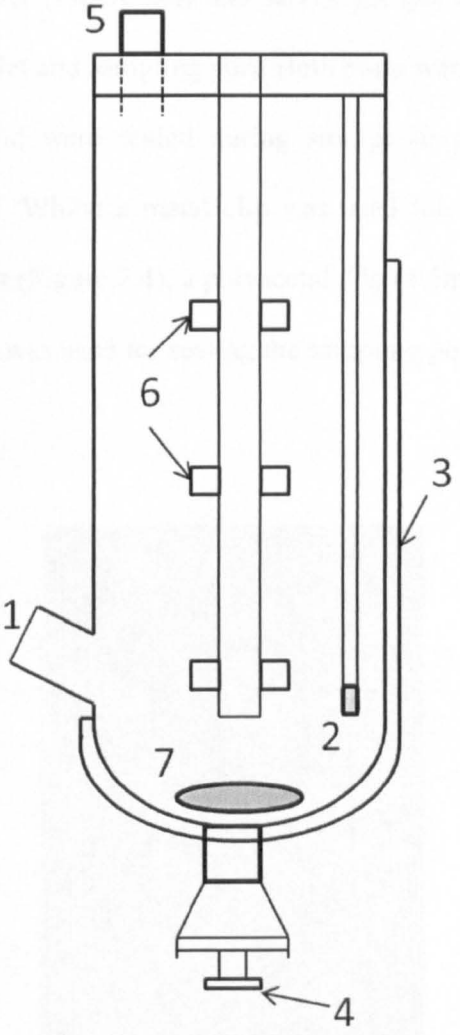



**Figure 2.3 - Schematic representation of an Infors bioreactor (modified from technical information sent by Infors, UK). 1 - Ingold port for installation of pH and DO probes, 2 - Temperature probe, 3 - external jacket for heating and cooling bioreactor, 4 - bottom sampling port, 5 - top port for media transfer and inoculation, 6 - baffles for stirring, 7 - circular dish located above the sampling port.**

## 2.5 Cropped Slurry Storage Vessels

Cropped slurry storage was performed in Duran vessels (Techfors-S, Infors HT, Switzerland), with a maximal working volume of 500 mL (refer to Figure 2.4). For maintaining anaerobiosis, each storage vessel was sealed with a 2-port stainless steel plate (Infors, HT, Switzerland). One of the ports was fitted

with a sterile gas filter (Figure 2.4) and served for gas inlet whilst the other served as the gas outlet and sampling port. Both ports were attached to silicone tubing extensions and were sealed during storage to prevent air (oxygen) ingress in the vessel. Whilst a metal clip was used for sealing silicone tube extension for gas port (Figure 2.4), a polyacetal clip (4.5mm diameter, Thermo fisher Scientifc, UK) was used for sealing the sampling port (seeFigure 2.4).

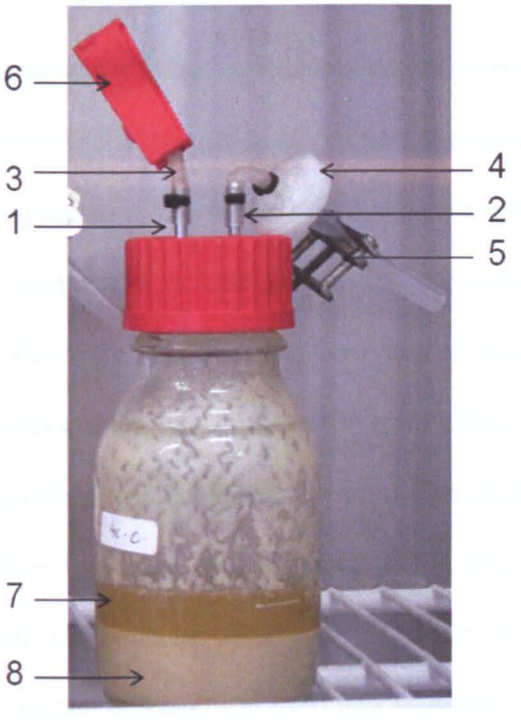



**Figure 2.4 - Yeast slurry storage vessel. 1 - sampling/gas outlet port, 2 - gas inlet port, 3 - silicone tube extension, 4 - sterile gas filter, 5 - metal silicone tubing clip, 6 - polyacetal silicone tubing clip, 7 - beer supernatant, 8 - sedimented yeast slurry.**

## 2.6 Cool Incubator Standardization

For the purpose of standardization of the cool incubator (MIR 253, Sanyo, Osaka, Japan), 100 mL of sterile, spent YPD was dispensed into mFVs coupled with thermometers (as described in Section 2.4.1) and maintained at

25 °C under continuous stirring (400 rpm) until attemperation had occurred. Stirring was achieved by placing the FVs on multiple place magnetic stirrer (MPMS) plates which were placed inside the cool incubator. Subsequently, the mini FVs were transferred to a cool incubator (MIR 253, Sanyo, Japan) set at 15 °C and the rate of reduction in temperature was measured. Assessments were conducted in triplicates.

## 2.7 Thermal Downshift Analysis using Yeast Pitching Densities

*W34/70* colonies from YPD agar slopes were propagated in sterile flasks and grown aerobically in wort for 3 days under continuous stirring (200 rpm) at 25 °C. Post centrifugation (3000 rpm, 5 mins, room temperature) the cell pellet was resuspended in spent wort to achieve a final cell concentration of $1.5 \times 10^7$ cells / mL. Following transfer of cell suspension, the mini FV-thermometer systems (Section 2.4.1) were maintained at the required initial temperature, in a cool incubator, under continuous stirring (400 rpm, using MPMS). Once the desired initial temperature was achieved, the mini FVs were transferred on a MPMS plate placed in a separate cool incubator (MIR 253, Sanyo, Japan) set at the desired final temperature. The rate of reduction in the media's temperature under constant stirring (400 rpm) was recorded.

## 2.8 Thermal Downshift Analysis using Cropped Slurry Densities of Yeast

Cropped yeast slurry was generated using step wise culture propagation by aerobic growth in 20 mL, 100 mL and 1 L sterile wort for 3 days each, at 25 °C under stirred conditions (400 rpm). Subsequently the cells were harvested from the growth media by centrifugation at 3000 rpm for 5-10 minutes at 25 °C. 30g of the obtained yeast pellet was resuspended either in 70 mL of (i) spent YPD,

(ii) spent wort or (iii) spent wort "beer" in FV-thermometer systems to obtain an overall yeast biomass concentration of 30% (w/v). Spent wort "beer" was prepared by addition of absolute ethanol to a final alcohol by volume (abv) of 7.6 % (v/v). The mFV-thermometer systems (Section 2.4.1) were then placed in a cooled incubator at the required temperature (MIR 253, Sanyo, Osaka, Japan) under stirred conditions (400 rpm, using MPMS). The analyses were conducted in triplicate.

## 2.9 Non-fermented yeast slurry storage

### 2.9.1 Yeast slurry formation

Representative colonies of *W34/70* were recovered from YPD agar slopes using a sterile loop and utilized for media inoculation. Step wise culture propagation was performed by aerobic growth in 10 mL (x6), 100 mL (x6) and 1 L (x6) sterile wort (15°P) for 48 hrs, 48 hrs and 72 hrs respectively at 25 °C with orbital shaking (120 rpm). Subsequently, cells were harvested by centrifugation at 3000 rpm for 5 min at 25 °C. 30g (wet weight) aliquots of the recovered yeast pellet were resuspended in an appropriate volume of spent wort "beer" (spent wort with ethanol; 7.6 % (v/v)) to obtain an overall yeast cell density of 30% (w/v).

### 2.9.2 Propagated Slurry storage

To simulate storage, freshly propagated yeast in the required media were dispensed in 15 mL aliquots into sterile universals (with small magnetic stirrers at the bottom), divided into three batches and simultaneously stored in incubators set to achieve a final temperature of 25 °C, 10 °C and 4 °C. Yeast aliquots were continuously stirred at 400 rpm using MPMS plates. The

universals were housed in containers in which anaerobic conditions could be maintained using Anaerogen $CO_2$ packs (Oxoid Ltd, Basingstoke, England). Maintenance of anaerobic conditions were assured by the use of anaerobic indicators (Oxoid Ltd, Basingstoke, England).

### 2.9.3 Sampling and cell preparation

Brewing yeast samples were harvested after 24, 48 and 72 hrs from each temperature condition (25, 10 and 4 °C) under a $CO_2$ purge in order to minimize any influx of external oxygen inside the containers. Samples were centrifuged at 3500 rpm for 5 min at room temperature and the "beer" supernatant was decanted. The resultant cell pellet was thoroughly resuspended in sterile deionised water (HPLC grade; 0.06 µS / cm) and further centrifuged at 3000 rpm for 5 min. This wash step was conducted twice to remove any beer debris adhering to the cellular surface. For analyses requiring live cell suspensions (such as cell viability determination and acidification power tests), an aliquot of washed yeast sample was recovered for immediate analysis whilst the remaining sample suspension was snap frozen in liquid nitrogen and stored at -20 °C for later assessment.

## 2.10 Serial re-pitching Experiments

### 2.10.1 Yeast Propagation

Representative *W34/70* colonies from agar slopes were propagated step wise (20 mL, 100 mL, 250 mL, and 1l) under aerobic conditions in sterile wort at 25 °C with orbital shaking (120 rpm). Except for the final propagation step (48 hrs), each of the previous stages lasted for 24 hrs. Cells were harvested and re-suspended in sterile water to obtain 50% (w/v) propagation slurry.

### 2.10.2 Pre-storage fermentation (G0F)

Propagation slurries were inoculated or "pitched" into 5.5 L of sterile wort in triplicate large-scale bioreactors (Techfors-S, Infors HT, Switzerland) (refer to Section 2.4.3). These represented pre-storage fermentations termed as G0F in this thesis. After thawing at room temperature, the fermentation broth or wort was first transferred into sterile Cornelius vessel and then asceptically into the fermentation vessels under air pressure. Prior to inoculation, wort was air saturated (5 L / min) through a rotameter under continuous mixing (400 rpm) for 12 hrs (overnight). Pitching was performed at a pitching rate of $1.8 \times 10^7$ cells / mL and during inoculation antifoam was also added (3-8 mL, 15%) (Sigma-Aldrich, UK). The fermentation vessels were sealed mechanically and the seal was lubricated with glycerine at all times i.e. during sterilization and actual fermentation.

Periodic aseptic sampling was performed from the bottom sampling port. Samples were immediately centrifuged (3500 rpm, 5 mins), the resultant yeast pellet was washed once with sterile water, flash frozen using liquid nitrogen and stored at -20 °C. Spent wort samples were aliquoted and stored frozen at -20°C until subsequent analysis.

### 2.10.3 Cropped Yeast Slurry Formation & Storage

The round bottomed Infors bioreactors were used to conduct G0F and therefore cone cropping was not permissible (since no cone was present in the vessels). As a result, an in-house laboratory based method was developed to achieve yeast sedimentation and slurry formation. Fermenting wort was transferred through the bottom sampling port (under nitrogen pressure) into

sterile 5-port graduated reaction vessels (capacity 2.5 L) (Figure 2.5) with magnetic stirrer at the bottom.

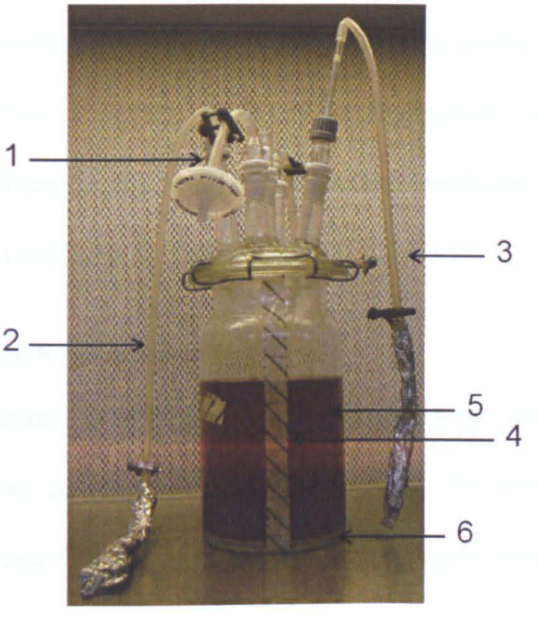



**Figure 2.5 - Five-port reaction vessel. 1 - gas port (one for inlet and one for exhaust), 2 - supernatant dispensing tube, 3 - slurry transfer tube, 4 - external gradations on the vessel, 5 - unsedimented supernatant and 6 - sedimented yeast slurry.**

Following transfer of fermenting media, the 2.5l reaction vessels were incubated in two cool incubators (Sanyo, Osaka, Japan) maintained at 10°C for further 24hrs, without stirring, to facilitate yeast sedimentation. The bulk of the unsedimented supernatant was then removed (under nitrogen pressure) (see Figure 2.5) leaving behind appropriate spent wort volumes to form 30% (w/v) of "cropped" brewing yeast slurry. Cropped yeast slurry was transferred and pooled (to maintain homogeneity) into a sterile, nitrogen-flushed 2 L Duran glass container and aliquoted (200 mL) into slurry storage vessels (described in Section 2.5) using nitrogen top-pressure.

Triplicate storage vessels were stored simultaneously in cool incubators set at either 4 °C or 10 °C. Sampling of stored yeast slurry was performed asceptically using sterile, stainless steel syringe needles (10 inch, 18 gauge) (Sigma Aldrich, UK) whilst flushing the headspace with nitrogen. Yeast slurry was washed once with sterile saline (0.8 % NaCl in sterile deionised water), flash frozen (-80 °C) and stored at -20 °C until required for analysis. Slurry volumes were maintained above 80 % of starting levels in order to minimize impact of volume variations on slurry status.

### 2.10.4 Post storage fermentation (G1F)

Following cold storage, *W34/70* slurry was harvested and pitched (inoculated) into 100 mL sterile, air-saturated hopped wort (14 °P) in mini fermentation vessels (mFVs) (described in Section 2.4.2). This post-storage fermentation has been termed as G1 fermentation (G1F). All G1Fs had an inoculum size of 1.8 x $10^7$ cells / mL, were continuously stirred (200 rpm) on MPMS plates and fermented simultaneously under identical conditions in cold-incubators. From each storage vessel, 7 mFVs were inoculated followed by removal of one mFV per storage vessel at each sampling point. This yielded triplicate G1F samples pitched with 10°C- and 4°C-stored slurries respectively. G1F samples were processed and stored in same manner as G0F samples (see Section 2.9.3).

## 2.11 Yeast Cell Viability and Budding Index

### 2.11.1 Cell viability using citrate methylene violet (CMV)

Citrate methylene violet (CMV) was utilized for determining yeast cell viability following the method of Smart *et al.* (Smart *et al.*, 1999). Accordingly, methylene violet 3RAX (Sigma-Aldrich, UK) was dissolved in

sodium citrate (2 % w/v in RO water) to a final concentration of 0.01% (w/v). 0.5 mL of live cell suspension ($1x10^7$cells / mL) was added to equal amounts of methylene violet and incubated (statically) at room temperature for 10 min. The cells were enumerated under a light microscope (name) using a haemocytometer. Stained cells were deemed non-viable whilst viable cells remained unstained. Viability was expressed as a percentage for triplicate analyses and a minimum of 200 cells were scored for each sample.

### 2.11.2 Cell Viability using Oxanol

Oxanol [bis-(1,3-dibarbituric acid)-trimethine oxanol; DiBAC4; Sigma-Aldrich, UK] (1mg) was dissolved in absolute ethanol to yield a stock solution of 1mg / mL. This was diluted 10 fold in sterile deionised water (90 µL) to serve as the working solution. Washed yeast cell suspension ($1x10^7$ cells / mL) was mixed with 0.1 mL oxanol working solution (final concentration 10 µg/ mL) and incubated in the dark at ambient temperatures for 10 min. Microscopic examination was conducted on a fluorescent microscope (Nikon OPTIPHOT; 400X magnification; Triple Pass Filter Set) where non-fluorescent cells were deemed viable and non-viable cells appeared lightly fluorescent. Viability was expressed as a percentage demonstrating the mean of three independent replicates. For each replicate, at least 200 cells were scored.

### 2.11.3 Budding Index

The percentage of budding cells within the yeast population was represented by the budding index (BI), determined using Equation 1.

$$BI = \frac{\text{number of cells exhibiting a bud}}{total\ number\ of\ cells} x\ 100$$

**Equation 1 – Determining budding index (BI) in a yeast population.**

### 2.11.4 Cell Density

Following dilution of cell suspension to appropriate cell numbers (around 1 x $10^7$ cells / mL), density of viable cell populations suspended in the media was calculated using a Neubauer counting chamber (Weber Scientific International Ltd, UK) and standard light microscope (400X; BH-2, Olympus, U.S.A.) using Equation 2. On each occasion 300 cells or more were scored.

$$\text{Cell Density (cells/ml)} = \frac{\text{cells} * \text{dilution factor}}{volume\ of\ chamber}$$

**Equation 2 – Calculation of cell density in yeast cultures using a haemocytometer.**

### 2.12 Acidification Power Test

Acidification power test (APT) was performed using the method of Kara *et al.* (1988) with modification from Siddique and Smart (2000). Washed, live cell suspensions were centrifuged to achieve a closely-packed cell pellet and stored under water for not more than 6 hrs before subsequent analysis. Immediately prior to measurement, yeast cell pellets were resuspended in sterile, deionised water (0.06 μS/cm) to obtain 1 x $10^9$ cells / mL. The calibrated pH probe (Inlab® Science Pro, Mettler Toledo, USA) was placed into a thin-walled universal with a magnetic flea and 19 mL of sterile deionised water. Following probe equilibration, 1 mL of yeast suspension was added and the initial pH ($pH_0$) immediately noted. The pH was recorded every 2 min for the next 10 min. At the end of 10 min, the pH was noted and immediately 5 mL of sterile

deionised water was added into the universal. The pH was monitored at 2 min intervals for a further 10 min ($pH20_{Water}$). The water acidification power (WAP or passive proton efflux) was obtained by subtracting the final pH from the initial pH (Siddique and Smart, 2000) as shown below (Equation 3a). Determination of the glucose acidification power (GAP; substrate-induced proton efflux) was similar to the method for WAP; however addition of sterile deionised water after 10 mins was replaced by 5 mL of 20.2% glucose (w/v). GAP was estimated as demonstrated in Equation 3b. The glucose-induced proton efflux (GIPE) was calculated by subtracting WAP from GAP (Equation 3c). Analysis was conducted in triplicates.

(a) $WAP = pH_0 - pH20_{Water}$

(b) $GAP = pH_0 - pH20_{Glu}$

(c) $GIPE = GAP - WAP$

**Equation 3 – Determination of (a) water acidification power (WAP), (b) glucose acidification power (GAP) and (c) glucose-induced proton efflux (GIPE). $pH_0$ - pH of cell suspension at the start of APT, $pH20_{Water}$ - pH after 20mins of water addition, $pH20_{glu}$ -pH after 20 mins with glucose addition.**

## 2.13 Intracellular Glycogen and Trehalose

Intracellular glycogen and trehalose were estimated based on modifications of the method of Parrou and Francois (Parrou and Francois, 1997). Frozen yeast samples were thawed on ice and an appropriate cell suspension volume containing 1 x $10^9$ cells was centrifuged at 3000 rpm for 5 min at 4°C. Cells were lysed by dissolving the pellet in sodium carbonate (0.5 mL; 0.25M) and incubated at 95°C for 2 hrs followed by the addition of sodium acetate (0.6 mL; 0.2M) and acetic acid (0.15 mL; 1M) to the cell suspension. 0.5 mL

aliquots were then removed into fresh eppendorf tubes. Glycogen and trehalose were broken down into glucose by adding 10 µl of α-amyloglucosidase (10 mg / mL; 59.9 units / mg; Fluka Biochemika, Steinheim) and trehalase (3 mUnits; Sigma-Aldrich, UK) respectively followed by incubation at 57°C and 37°C for 14 hrs. Post incubation, samples were centrifuged (3500 rpm; 5 min) and the supernatant (0.1 mL) used for glucose quantification using the Megazyme Glucose Assay kit (GOPOD, Megazyme, Ireland) at an optical density of 510 nm against glucose standards (1, 0.5, 0.4, 0.25 and 0.1 mg / mL). Analysis for each time point was conducted in triplicates and results were expressed in concentration of glucose as a function of cell number or dry cell weight.

### 2.14 Intracellular Fatty Acid

Intracellular fatty acids (FA) were analyzed from cell suspensions containing 1 x $10^9$ cells using GC-MS. After boiling (95°C; 5 min) and centrifugation (13000rpm; 1 min), the cell pellet was resuspended in 0.5 mL Hexane-Propanol (2:1, v/v) containing methyl heptadecanoate as the internal standard (0.252 mg / mL). The cell suspension was homogenised using bead milling (0.5mm, Biospec products Inc., USA; 3 x 45 sec) and after centrifugation (13000 rpm; 1 min) the upper organic phase was transferred into clean microfuge tubes. Following washing with distilled water (500 µl, 0.06 µS / cm), the supernatant was further removed into fresh microfuge tubes and dried with nitrogen. The lipid fraction was dissolved in diethyl ether (0.5 mL) and transferred into GC vials. After drying the ether using nitrogen and heating (75 °C; 20 min), the extracted lipids were dissolved in chloroform (0.5 mL) and derivatisation was performed using Trimethylsulphoniumhydroxide (TMSH)

(40 µl, 0.25 M; Sigma-Aldrich, UK) at room temperature (15 min) to generate fatty acid methyl esters (FAMEs).

The methyl esters were then analyzed using gas chromatography coupled with mass spectrometry. 1 µL aliquots of the samples were injected in split mode (1:20) using an AS 3000 autosampler (Thermo Scientific, Massachusetts) into the injector port (250 °C) of a Trace GC ULTRA (Thermo Scientific, Massachusetts) fitted with a ZB-Wax column, 30m x 0.25mm and ID 1µm film thickness (Phenomenex, Macclesfield, UK). The initial oven temperature was maintained at 120 °C for 1 min after injection, then ramped at 5 °C / min to 250 °C and finally maintained at 250 °C for 13 min. Column head pressure was maintained at 18 psi (helium carrier gas). The compounds were detected using a DSQ II mass spectrometer (Thermo Electron Corporation, Thermo Scientific, Massachusetts) operating in selected ion mode. The ions monitored were m/z 74, 87, 91 and 97 with a dwell time of 0.05 sec. Chromatographic peak identification was performed using an external standard supplied by Sigma (Supelco 37 Comp. FAME Mix, Steinheim, Germany). The peak areas for the various FAs were quantified using the Xcalibur software (Thermo Fisher Scientific, UK). Quantified peak areas were then normalized with that of the internal standard (using Microsoft Excel 2007, USA). The order of peaks in the chromatogram along with retention times in the GC column have been mentioned in Table 2-4. The formulae used for the determination of total long-chain saturated (SFA) and unsaturated (UFA) fatty acid alongside percentage Medium Chain Fatty Acid (MCFA) and Unsaturation Index (UI) have been mentioned in Equation 4.

| FA Common Name | FA Common Nomenclature | Retention Time (mins) |
|---|---|---|
| **Non-fermented Yeast Storage** | | |
| Palmitic Acid | C16:0 | 22.2 |
| Palmitoleic | C16:1 | 22.8 |
| Heptadecanoic Acid | C17:0 | 24.19 |
| Stearic Acid | C18:0 | 26.12 |
| Oleic Acid | C18:1 | 26.58 |
| **Fermented Yeast storage** | | |
| Capric Acid | C10:0 | 8.09 |
| Lauric Acid | C12:0 | 12.2 |
| Myristic Acid | C14:0 | 16.5 |
| Palmitic Acid | C16:0 | 20.7 |
| Palmitoleic | C16:1 | 21.3 |
| Heptadecanoic Acid | C17:0 | 22.69 |
| Stearic Acid | C18:0 | 24.61 |
| Oleic Acid | C18:1 | 25.07 |

**Table 2-4 - List of fatty acids (FA) analyzed during yeast storage. Non-fermented yeast and fermented yeast samples were generated as mentioned in Section 2.9.2 and Section 2.10.3 respectively. The order of FA peaks in the MS chromatograms is the same as depicted above. Retention time for the utilized ZB-Wax Column has also been mentioned.**

(a) $SFA = \sum(C14{:}0 + C16{:}0 + C18{:}0)$

(b) $UFA = \sum(C16{:}1 + C18{:}1)$

(c) $\mathrm{UI} = \mathrm{SFA}/UFA$

(d) $MCFA = \sum(C10{:}0 + C12{:}0$

**Equation 4 - Formulae representing the calculation of various parameters for estimating overall fatty acid distribution in stored yeast populations. SFA - total long-chain Saturated Fatty Acid, UFA - total long chain Unsaturated (UFA) Fatty Acid, UI Unsaturation Index, MCFA - total Medium Chain Fatty acid. C10:0, C12:0, C14:0, C16:0, C16:1, C18:0 and C18:1 represents different fatty acids as per the nomenclature defined in Table 2-4.**

## 2.15 Intracellular Glycerol

Intracellular glycerol determination was conducted as reported previously (Wojda *et al.*, 2003). Snap frozen cell pellet containing 1 x $10^9$ cells (and washed once with 0.8% NaCl prior to freezing) (see Section 2.10.3) were thawed on ice, resuspended in 1 mL boiling Tris-HCl and heated in a boiling water bath for 10 mins. The lysed cells were centrifuged at 4000 rpm for 10mins (room temperature) and the supernatant was transferred to fresh microcentrifuge tubes. 0.1 mL of the supernatant was then assayed for glycerol content using a commercial kit (Megazyme International Ireland Ltd., Ireland). This involved pipetting 2 mL distilled water, 0.1 mL sample, 0.2 mL solution 2 (NADH/ATP/Phosphoenolpyruvate buffer) and 0.02 mL suspension 3 (Pyruvatekinase and Lactate dehydrogenase buffer) in a cuvette followed by agitation and absorbance measurement ($A_1$) at 340 nm against water after 4 mins. Afterwards, 0.02 mL of Suspension 4 (Glycerokinase solution) was added to the same cuvette, mixture was mixed and absorbance recorded ($A_2$) again at 340 nm after 5 mins. The concentration of glycerol was then calculated using Equation 5 and has been represented as a function of cell dry weight for triplicate analyses. A glycerol standard (0.2 mg / mL) supplied with the kit was used for ensuring assay consistency.

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times (A2 - A1)$$

**Equation 5 - Formula for glycerol determination using the glycerol assay kit as provided by Megazyme International Ltd, Ireland. c - glycerol concentration in g/L, V - final volume (mL), MW - molecular weight of glycerol (g / moL), ε - extinction coefficient of NADH at 340 nm, d - light path (cm), v - sample volume (mL).**

## 2.16 Transcriptome analysis using Microarrays

### 2.16.1 RNA Extraction

Previously washed and frozen yeast pellet (see Section 2.10.3) containing 1 x $10^8$ cells was thawed on ice and subjected to RNA extraction using RiboPure-Yeast Kit (Ambion, Applied biosystems, UK). Unless otherwise stated all reagents and other components were supplied with the kit. Following manufacturer's instructions, 750 µL of cold Zirconia beads were dispensed into a screw cap tube (1.5 mL) followed by addition of yeast cell suspension previously resuspended in a mixture of lysis buffer (480 µL), 10% SDS (48 µL) and Phenol:Chloroform:IAA (480 µL). The cells were then lysed using a vortex adapter, centrifuged (14000 x g, room temperature) for 5 mins and the upper aqueous phase (approximately 500 µL) containing partially purified RNA was transferred to a fresh 4-15 mL capacity tube. To this aqueous extract, 1.9 mL binding buffer and 1.25 mL absolute ethanol (Thermo fisher Scientific, UK) was added with intermittent agitation ensuring thorough mixing. Using a 5 mL syringe, the sample mixture was then applied to a filter cartridge assembled in a collection tube in several applications (around 700 µL per application) followed by centrifugation (13000 x g, 30 sec, room temperature) after each application. Once the entire sample was passed through the filter cartridge, it was washed once with wash solution 1 (700 µL) and twice with wash solution 2/3 (500 µL each time) with intermittent centrifugation (13000 x g, 30 sec) after each wash step. Following another centrifugation step (13000 x g, 1 min) the bound RNA was subjected to two sequential elutions with 50 µL of elution solution at each step. An aliquot (780 µL) of the RNA thus extracted was assembled in a fresh microfuge tube with 10X DNase 1 buffer (8 µL), 4

μL DNase 1 (8 units) and incubated at 37 °C for 30 mins. The mixture was then treated with 9 μL of DNase inactivation agent, mixed by vortexing and incubated at room temperature for 5mins to stop the enzymatic reaction. The suspension was centrifuged at 13000 x g for 3 mins at room temperature to pellet the inactivation reagent and the pure RNA-containing supernatant was transferred to a fresh tub.

### 2.16.2 RNA hybridization and Microarray data acquisition

The purified RNA (Section 2.16.1) was assessed for purity and concentration using NanoDrop® (ND-1000, Thermo Scientific) and by microcapillary electrophoresis (2100 Bioanalyzer, Agilent Technologies, Inc.) at the European Arabidopsis Stock Centre (NASC, University of Nottingham). Sample preparation, hybridization to yeast genome 2.0 array and scanning was performed as per manufacturer's instructions (GeneChip Expression Analysis, Affymetrix) by NASC. Following scanning, the GeneChip command console (AGCC, Affymetrix Inc., USA) was used to generate non-scaled RNA (.cel) files. These files contained signal intensity values from each of the thousands of probe sets on GeneChip 2.0 array, each consisting of 11 pairs of perfect match and mismatch probes.

The non-scaled RNA .cel files provided by NASC were loaded into the GeneSpring GX 11 (Agilent Technologies, USA) software and pre-normalized as a single experimental group using the Robust Multichip Average (RMA) algorithm. This summarised the signal values in log scale using the statistical method, Median Polish (Irizarry *et al.*, 2003). The signal value for each probe-set was further normalised using the per gene normalisation strategy. This involved standardising the baseline of a gene to the median signal value (for

that particular probe) for all arrays included in that analysis. One-way ANOVA ($p < 0.05$) was utilized to identify difference in hybridization intensities between different time points and test/reference samples at a particular sampling point.

### 2.16.3 Probe selection using XSpecies

Due to lack of commercial availability of *S.pastorianus* chips, the Xspecies or cross species hybridization approach was adopted (Chaturvedi *et al.*, 1997, Albertyn *et al.*, 1994a). For the same, genomic DNA extract (described in Section 2.3.3) was labelled using Bioprime DNA labelling system (Invitrogen) and hybridised to Genome 2.0 GeneChip array following standard manufacturer protocols (Affymetrix). The arrays were scanned using a 3000 7G Gene Array scanner (Affymetrix) and gDNA hybridisation intensity (.cel) files were generated using the GeneChip command console (AGCC, Affymetrix Inc., USA). Probe-pairs from the gDNA hybridisation intensity files were selected using a probe masking file (.cdf) written in Pearl script and available from www.affymetrix.arabidopsis.info/xpecies. The mask file specifically contains those probe-pairs in which the perfect match probe has a gDNA hybridisation intensity greater than the user defined threshold (Hammond et al., 2005). Hybridisation threshold intensities were set at 0, 20, 40, 60, 80, 100, 150, and 200.

## 2.17 Determining Ethanol and Gravity

Alcohol and specific gravity determination of spent wort samples were performed simultaneously with an Alcolyzer-Plus system attached to a DMA 4500 density meter (Anton Paar Ltd., U.K.). The Alcolyzer measured ethanol amounts by near infrared (NIR) absorption (between wavelengths of 1150 to

1200 nm) and yielded alcohol by volume (ABV) measurements. The density meter determined specific gravity using an oscillating u-tube. Wort or fermentation broth samples were first thawed at room temperature followed by centrifugation (3000 rpm, 5mins, 4°C) for trub and cell debris removal. Analysis was conducted using distilled water as control (according to manufacturer's instructions).

### 2.18 Determining Fermentable Sugars

Quantification of the four major fermentable sugars namely glucose, fructose, maltose and maltotriose in spent wort was performed using HPLC (Gibson *et al.*, 2008). Wort samples (1 mL) were combined with 100 µl of melizitose solution (100 mg/ mL) as the internal standard (IS). These were passed through solid phase extraction cartridges (Strata-X, 33 µg, Phenomenex, U.K.) previously subjected to methanol (1 mL) conditioning and water (1 mL) equilibration. Post clean-up, samples were collected in glass bijous and 5µl was injected onto an amino column (250 mm x 2 mm i.d., Luna $NH_2$ with 5 µm particle size, Phenomenex, U.K.) with sugars eluted using degassed acetonitrile : water (80:20, v/v) at a constant flow rate (0.7 mL/min) with a typical back pressure reaching 2200 psi using an HPLC liquid pump (Pump PU-980, Jasco, Japan). Eluent detection was performed using a Refractive Index Detector (Jasco, USA) and Azur Chromatography software was utilized for peak area quantification. Ratios of target compound to IS (melizitose) peak areas in sugar standards (with known concentrations) were calculated to generate linear calibration curves and utilized for calculating spent wort sugar concentrations. The elution order and retention times for the various sugars

were – fructose (2.18 min), glucose (2.53 min), maltose (4.12 min), melezitose (5.67 min) and maltotriose (6.75 min).

### 2.19 Determining Free Amino Nitrogen (FAN) content

Free amino nitrogen (FAN) in spent wort was determined using the standard ninhydrin-based method as defined by the American Society of Brewing Chemists Methods of Analysis (ASBC, 1992). Barring proline, the method quantifies amino acids, ammonia and to a certain extent end group α-amino nitrogen in proteins and peptides (ASBC, 1992). A glycine stock solution was prepared by dissolving 107.2 mg of glycine (Sigma-Aldrich, UK) per 100 mL of distilled water. This was subsequently diluted 1 part in 100 in water to generate a working glycine standard solution containing 2 mg amino nitrogen / L of water (since 1 mg of glycine contains 0.186 mg of nitrogen). Ninyhydrin colour reagent was produced by dissolving 10g of sodium phosphate dibasic dodecahydrate ($Na_2HPO_4.12H_2O$) (Sigma–Aldrich, U.K.), 6g of potassium phosphate monobasic ($KH_2PO_4$) (Sigma–Aldrich, U.K.), 0.5g of ninhydrin (Sigma–Aldrich, U.K.) and 0.3g of fructose (Fisher Scientific, UK) in 100 mL distilled water. In a sterile test tube, each wort or fermentation sample was diluted 1:100 (20ul in 2 mL) in distilled water followed by addition of ninhydrin colour reagent (1 mL). The test tubes were capped, heated in a boiling water bath (16 mins) and cooled in a separate water bath maintained at 20 °C (20 mins). 5 mL of dilution solution, prepared by dissolving 2g of potassium iodate ($KIO_3$) (Sigma-Aldrich, UK) in 600 mL of distilled water and 400 mL of 96% ethanol, was added. The contents of each test tube were mixed thoroughly and the absorbance of the solution was measured at 570 nm against distilled water. FAN was calculated using Equation 6. All samples and

standards were analyzed in triplicates with fresh standard prepared every time prior to analysis.

$$\text{Free Amino Nitrogen (FAN)} = \frac{\text{Net absorbance of test solution}}{\text{Net absorbance of glycine standard}} \times 2 \times 100$$

**Equation 6 – Calculation of free amino nitrogen (FAN) (ASBC, 1992).**

### 2.20 Determining Amino Acid content

Isolation and derivatization of amino acids in wort samples was conducted using the EZ:FAAST amino acid kit (Phenomenex, Macclesfield, UK) (Gibson *et al.*, 2009). 25 µl of sample was mixed with 20 nmol of norvaline (IS) and passed through a solid phase extraction absorbent (contained within a pipette tip) which was subsequently washed with 200 µl propanol. The absorbent (including the retained amino acids) was removed from the pipette tip by a solution of propanol and sodium hydroxide (200 µl). Derivatization of the amino acids was achieved by sequential addition of chloroform (50 µl) and iso-octane (100 µl) following which the amino acids were recovered in the upper organic layer. This was dried down under a nitrogen stream and the sample redissolved in 100 µl of Iso-octane:chloroform (80:20 v/v). For subsequent analysis, 1 µl of sample was injected in splitless mode using an AS3000 autosampler (Thermo Fisher Scientific, UK). The injector of the Trace GC Ultra gas chromatograph (Thero Fisher Scientific, UK) was maintained at 250 °C, with an initial oven temperature of 90°C followed by gradual increments at 20 °C / min to 320 °C. Helium served as the carrier gas (8 psi) whilst amino acid separation was performed on a ZB-AAA column (10m x 0.25mm I.D.) supplied with the kit. The mass spectrometer (DSQ II, Thermo Fisher Scientific, UK) was operated in selected ion mode recording ions as listed in

Table 2-5 with a dwell time of 0.03s. Standard solutions were prepared as per the manufacturer's instructions at 2, 5, 10 and 20 nmols per unit (each unit corresponding to 100 μl of IS). After adjustment for IS peak area variations (normalization of amino acid areas with IS peak area), calibration curves were prepared for the amino acids detected in the standards and the graph's (linear) gradient was subsequently utilized for amino acid quantification within the wort samples.

**Table 2-5 - The amino acids detected following GCMS analysis. The column retention time and ion used for quantification for each amino acid is indicated.**

| Amino acid | Acronym | Retention time (min) | Ion for Quantification |
|---|---|---|---|
| Alanine | ALA | 2.07 | 130 |
| Sarcosine | SAR | 2.19 | 130 |
| Glycine | GLY | 2.26 | 144 |
| Abscisic acid | ABA | 2.44 | 144 |
| Valine | VAL | 2.6 | 158 |
| β-aminoisobutyric acid | B-AIB | 2.71 | 116 |
| Norvaline (IS) | NOR | 2.83 | 158 |
| Leucine | LEU | 2.97 | 172 |
| allo-Isoleucine | AILE | 3 | 172 |
| Isoleucine | ILE | 3.05 | 172 |
| Threonine | THR | 3.4 | 101 |
| Serine | SER | 3.46 | 146 |
| Proline | PRO | 3.54 | 156 |
| Asparagine | ASN | 3.73 | 155 |
| Aspartic acid | ASP | 4.63 | 130 |
| Methionine | MET | 4.66 | 101 |
| 4-Hydroxyproline | 4HYP | 4.88 | 172 |
| Glutamic Acid | GLU | 5.2 | 172 |
| Phenylalanine | PHE | 5.21 | 146 |
| α-Aminoadipic acid | AAA | 5.69 | 184 |
| Glutamine | GLN | 6.18 | 84 |
| Ornithine | ORN | 6.84 | 156 |
| Lysine | LYS | 7.25 | 116 |
| Histidine | HIS | 7.54 | 180 |
| Tyrosine | TYR | 7.98 | 116 |
| Tryptophan | TRP | 8.41 | 130 |
| Cystine | C-C | 9.56 | 248 |

IS – Internal Standard

## 2.21 Determining Flavour metabolites

### 2.21.1 Vicinal diketone (VDK)

The amounts of VDK present in wort or fermentation broth samples were assessed following the method of Jenkins D. (Jenkins, 2011). Accordingly,

diacetyl standard of 25 ppm was prepared in 5% (v/v) ethanol (Fisher Scientific, UK) followed by 1:2 serial dilution to generate a standard series containing 25, 12.5, 6.25, 1.56, 0.78 and 0.19 ppm diacetyl. A 250 ppm stock solution of the internal standard 1,2-dichloropropane (Sigma-Aldrich, UK) was prepared in absolute ethanol, which was subsequently diluted 1:50 with water to produce a working solution. To 5 mL of thawed sample (wort or fermentation broth), 3.5g of ammonium sulfate (Sigma-Aldrich, UK) and 100 µL of IS was added and the mixture agitated. An aluminium crimp seal with a pre-fitted septum (Thermo Fisher Scientific, UK) was used to seal the vial. The vials and its contents were heated for 30 min at 70 °C in a GC oven (Thermo Fisher Scientific), allowed to cool at room temperature and placed in the tray of the combiPal autosampler (CTC Analytics, Switzerland). The samples were equilibrated at 45°C for 5 min with agitation at 500 rpm, followed by 1 mL of head space injection into the gas chromatograph (Trace GC Ultra, Thermo Fisher Scientific, UK). The syringe temperature was set at 60°C whilst the gas chromatograph injector was kept at 125°C. The oven temperature was maintained at 40°C for 2 mins with subsequent increments of 10 °C / min to a final temperature of 130°C. At a constant flow rate of 1 mL/min, Helium was utilized as the carrier gas whilst a ZB-Wax column (30m x 0.25mm x 1µm) (Phenomenex, UK) was used for separation of investigative compounds. A DSQ mass spectrometer (Thermo Fisher Scientific, UK) was operated in selected ion mode (ions detected - 43 and 86) with a dwell time of 0.45 sec. The retention time for the diacetyl peak was 7.15 mins when detected using both 43 and 86 ions.

### 2.21.2 Esters and Fusel alcohols using Solvent-Extraction GCMS

Metabolite analysis using solvent extraction coupled with GC-MS was performed according to the method of Ashraf *et al.* (2010) with slight modification.

#### 2.21.2.1 Calibrant preparation

A calibrant stock solution containing 50 ppm of isoamyl alcohol, active amyl alcohol, 2-phenyl ethanol, ethyl octanoate and 2-phenyl ethyl acetate was prepared by adding 50 µL of constituent chemicals to 1L of 5% (v/v) ethanol solution (see Table 2-6 for manufacturer information). Following agitation, the stock solution was diluted, using 5% (v/v) ethanol, at ratios of 1:2, 1:5, 1:10, 1:100 and 1:1000, to obtain a calibration series containing 50ppm (stock), 25ppm, 10ppm, 5ppm, 0.5ppm and 0.05ppm of individual metabolites. The calibrant solutions thus generated were then extracted and analyzed in the same way as the test samples. An internal standard (IS) stock solution at 250 ppm was also prepared by addition of 25 µL of 3-heptanone to 100 mL methanol followed by gentle agitation.

| No. | Metabolites | Source | Retention Time (mins) | Quantifying Ion (m/z) |
|---|---|---|---|---|
| **Solvent Extraction - GCMS** | | | | |
| 1 | Isoamyl alcohol | SAFC, Sigma-aldrich, USA | 5.83 | 42 |
| 2 | Active amyl alcohol | Sigma-Aldrich, Germany | 5.92 | 56 |
| 3 | 3 - Heptanone (IS) | Sigma-Aldrich, Germany | 9.2 | 57 |
| 4 | 2-Phenyl Ethanol | Sigma-Aldrich, Germany | 14.3 | 122 |
| 5 | Ethyl octanoate | Sigma-Aldrich, Germany | 15.67 | 88 |
| 6 | 2-Phenyl ethyl acetate | SAFC, Sigma-Aldrich, Germany | 17.02 | 104 |
| **Head Space - GCMS** | | | | |
| 1 | Acetaldehyde | Acros Organics, USA | 2.24 | 44 |
| 2 | Ethyl acetate | SAFC, Sigma-Aldrich, Germany | 5.74 | TIC |
| 3 | Ethyl propionate | Sigma-Aldrich, Germany | 7.88 | 56 |
| 4 | 2-Butanol (IS) | Sigma-Aldrich, Germany | 9.8 | 59 |
| 5 | Ethyl butyrate | SAFC, Sigma-Aldrich, Germany | 10.1 | 43 |
| 6 | n-propanol | SAFC, Sigma-Aldrich, Germany | 10.56 | 59 |
| 7 | Isobutanol | SAFC, Sigma-Aldrich, Germany | 11.17 | 43 |
| 8 | Isoamyl acetate | SAFC, Sigma-Aldrich, Germany | 11.77 | TIC |

**Table 2-6 - List of compounds used in calibrant preparation for detection of flavour metabolites using solvent extraction-GC-MS and headspace-GC-MS. The manufacturer (source) of the compounds along with their retention times and the ions used for quantification have also been shown.**

### 2.21.2.2 Sample preparation

250 µL of internal standard was simultaneously added to 5 mL aliquots of wort samples and calibrant series solutions; final IS concentration was at 12.5 ppm. Following gentle agitation, sample-IS mixture was extracted with 1 mL dichloromethane (DCM) through mixing on a roller-bed for 1 hour at room temperature (Ashraf *et al.*, 2010). After spinning the mixture at 1800 rpm for 5min (at 4 °C), the lower dichloromethane layer was collected in 0.3 mL GC glass vials and stored at -20 °C until subsequent GC-MS analysis.

### 2.21.2.3 GC-MS Analysis

Using an AS 3000 autosampler (Thermo fisher Scientific, UK), 1 µL aliquots of the dichloromethane extracts were injected into a Trace GC Ultra's injector port, maintained at 250 °C, in splitless mode. The initial oven temperature was maintained at 40 °C for 1 min after injection with consequent increments of 8

°C / min to a final temperature of 250 °C. Compounds were eluted through a ZB-5 column, 30m length × 0.25mm i.d × 1 µm thickness (Phenomenex, UK) under 18 psi column head pressure using Helium as the carrier gas. Compound detection was performed on a DSQ II mass spectrometer (Thermo Fisher Scientific, UK) operating in full scan mode (m/z 30 - 250) at 2 scans / sec.

### 2.21.3 Esters and fusel alcohols using Head space GCMS

#### 2.21.3.1 Calibrant preparation

A stock calibrant solution preparation containing 50 ppm concentrations of acetaldehyde, isobutanol, n-propanol, isoamyl acetate, ethyl propionate, ethyl acetate and ethyl butyrate was prepared in 1L of 5% (v/v) ethanol (see Table 2-6 for manufacturer information). Subsequently, the stock solution was diluted at ratios of 1:2, 1:5, 1:10 and 1:100 to generate a calibrant series with flavour metabolite concentrations of 50, 25, 10, 5 and 0.5 ppm respectively. 2-Butanol stock (250 ppm) prepared in absolute methanol was used as the IS.

#### 2.21.3.2 Sample preparation

For head space GC-MS, 5 mL aliquots of the sample (fermentation broth samples or calibrant solutions) were dispensed in 20 mL headspace vials (DIN crimp neck, round bottom, Thermo Fisher Scientific, USA). After the addition of 250 µL of 2-Butanol stock solution (final concentration at 12.5 ppm) the vials were sealed tightly with magnetic aluminium crimps containing a pre-fitted PTFE silicon septum (Thermo Fisher Scientific, USA).

#### 2.21.3.3 Head Space Injection

Using a Combi PAL autosampler (CTC Analytics AG, Switzerland) 1 mL of sample head space was injected in splitless mode (split flow at 30 mL / min)

into the sample port of a Trace GC Ultra (Thermo Fisher Scientific, USA). Injection was performed using a gas tight syringe (2.5 mL maximum volume) (CTC Analytics AG, Switzerland) with the injector port being maintained at 220°C. A ZB Wax column (30m × 0.25 mm × 1 μm, Phenomenex, Macclesfield, UK) was used for compound separation using helium as the carrier gas at a constant flow rate of 1.5 mL/min. The oven temperature was maintained at 30 °C for the first 5 min, increased at the rate of 10 °C/min for the next 10 min and held at 100°C for 4.5 mins. A DSQ mass spectrometer operating in select ion mode recorded single ion chromatograms at ions (m/z) 15, 29, 31, 41, 42, 43, 44, 45, 46, 56, 71 and 72.

### 2.21.4 GC-MS Data Acquisition and Analysis

For all sets of flavour metabolite analysis, the peaks in the chromatograms produced were detected and manually assigned using Xcalibur software (Thermo Fisher Scientific, UK). Following normalization, peak areas of the calibrant solutions were calculated and plotted against known calibrant concentrations to generate standard curves for quantification of the various flavour compounds.

# CHAPTER 3: STRAIN CHARACTERIZATION & YEAST THERMOTOLERANCE ASSESSMENT

## 3.1 Introduction

Over 100 genera representing 700 different species of yeast have been reported (Boulton and Quain, 2001). By no means does this statistic represent the complete picture of yeast diversity and new genera and species are discovered every so often making yeast taxonomy highly complex and, at times, controversial.

Despite close taxonomical relations, different strains of brewing yeasts have evolved through selection and have become adapted to specific applications. The use of *Saccharomyces cerevisiae* strains for brewing ale-type beer has been established for centuries (starting as early as 6000 BC) (Spellman *et al.*, 1998). In contrast, lager beer, with its characteristic lower temperature (5-14 °C) fermentation regimes, is of comparatively recent origin; probably arising in Bavaria towards late Middle Ages (Tehlivets *et al.*, 2007). The "cooler" alcoholic beverage started gaining global popularity towards the early 19$^{th}$ century coincident with the advent of refrigeration facilitating maintenance of cool fermentation temperatures all year round. There is a wealth of evidence suggesting that different brewing isolates (from different geographic locations) can encompass diverse combinations of the closely related *Saccharomyces sensu stricto* species (Casaregola *et al.*, 2001) (Zhang *et al.*, 1995) (Dombek *et al.*, 1999) (Smart, 2007) (Tehlivets *et al.*, 2007). However, the lager yeast *S.pastorianus* is both physiologically and genetically distinct from *S.cerevisiae* (Boulton and Quain, 2001) (Tehlivets *et al.*, 2007).

In most breweries that simultaneously use lager and ale yeasts, periodic checks of yeast stocks is imperative to ensure absence of cross-contamination and intact genetic homogeneity. As a consequence of the utilization of a large number of production yeast strains which fail to conform to a particular physiological or genetic characteristic on account of their inherent natural divergence, a single test capable of unambiguous identification of yeast strains is still elusive (Boulton and Quain, 2001). Thus, a multitude of approaches have been developed for differentiating brewing yeast strains. Most of these differentiating methods can be broadly classified into traditional or modern methods (Boulton and Quain, 2001). Whilst the former are primarily based on conventional brewing microbiology, the latter are highly influenced by our improved understanding of yeast molecular biology in recent years.

### 3.1.1 Ale and lager strain differentiation based on maximal growth temperature

Perhaps the simplest method of differentiating ale and lager yeast strains is to exploit the difference in temperature required for achieving maximal growth rates. Ale strains, along with wild yeasts, require a higher temperature (37.5 to 39.8 °C) for attaining their maximum growth potential when compared to lager strains (31.6 to 34 °C)(Boulton and Quain, 2001). This test is one of the traditional methods and is routinely utilized in the brewery Quality Assurance laboratories for strain differentiation at a rather superficial but effective level.

### 3.1.2 Ale and lager strain differentiation based on melibiose utilization

The ability of lager strains to utilize the disaccharide melibiose is well known and has long been used by taxonomists and yeast physiologists for differentiating ale (lacking melibiose utilization capacity) and lager strains.

The principle enzyme involved in melibiose utilization is α-galactosidase, which is widespread in mycelia fungi, plants and animals but relatively uncommon in yeast (Zaragoza *et al.*, 2002, Daran *et al.*, 1997). The enzyme can hydrolyze the disaccharide melibiose into galactose and glucose which in turn could be utilized for cell growth. The activity of α-galactosidase is tightly regulated by galactose (induction) and glucose (repression) (Bell *et al.*, 1998). The occurrence of genes encoding α-galactosidase (named as *MEL* genes) in yeast's *cerevisiae* species is highly varied (Hwang *et al.*, 1989). Although the bulk of the *cerevisiae* strains lack any of the *MEL* genes (Francois and Parrou, 2001), others with the melibiose assimilating phenotype can potentially contain as many as 11 structural α-galactosidase genes, designated *MEL1-MEL11* (Bell *et al.*, 1992) (Van Dijck *et al.*, 2002), in various combinations. Like most gene families identified in yeast, the *MEL* gene family demonstrate high identity between nucleotide sequences (96 - 100%) and the constituent genes were mapped in the telomeric regions of 11 different chromosomes (Zaragoza *et al.*, 2001).

### 3.1.3 Ale and lager strain differentiation based on genomic sequence variation

As alluded to before, lager and ale yeasts exhibit fundamental differences in their genomic constitution. The genome of ale-producing yeast strains (usual taxonomic representation being *S.cerevisiae*) is highly diverse due to long-term geographical and environmental adaptation and lacks any sort of common DNA fingerprint (Cheng *et al.*, 1995). On the other hand, bottom-fermenting lager yeasts essentially contain hybrid genomes with a diverse range of

contributions from *S.cerevisiae*, *S.bayanus* and *Saccharomyces bayanus var. Uvarum* (Rowen *et al.*, 1992) (Dunn and Sherlock, 2008).

Based on the considerable genomic diversity prevalent between lager- and ale-producing yeasts, a PCR-based method was previously developed in our laboratory which can be used for differentiation between lager and ale forming yeast species. Since, the technology is currently part of a patent application the exact details cannot be disclosed (Patent Application No. 09785402.0, Ex PCT/GB2009/050928). To give a brief overview, one of the exons in the genome of ale and wild-type yeasts is split in lager brewing yeasts by an intervening intron sequence. As a result primers designed to amplify the intact exon in ale and wild-type strains would yield a shorter DNA fragment as opposed to a larger amplicon size encompassing the split exon moieties in lager strains.

## 3.2 Results

In this study the principle lager yeast strain that has been studied is a *Saccharomyces cerevisiae* (*Saccharomyces syn. pastorianus*) lager yeast strain denoted W34/70. This strain is widely used by the brewing industry and was therefore selected as the principal strain for assessment. In order to confirm the lager strain phenotype of W34/70, a blend of traditional and modern methods were utilized. Three different parameters were deployed based on: (i) maximum growth temperature; (ii) capacity to utilize metabolism; and (iii) genomic DNA sequence. Control strains of lager and ale *Saccharomyces* species denoted NCYC 1116 and NCYC 2593 respectively were also utilized.

### 3.2.1 Permissive Growth Temperature

To assess the permissive growth temperature of W34/70 and the control strains, representative colonies were streaked onto agar plates and inoculated at 25 °C, 34 °C and 37 °C according to the method outlined in Section 2.3.1 (Chapter 2). The ale strain, NCYC 2593, demonstrated optimal growth at 34°C and 37°C whereas both of the lager strains, W34/70 and NCYC 1116, demonstrated sparse growth at 34°C and no growth at 37°C (see Figure 3.1 - B and C). All strains grew well at 25 °C (Figure 3.1 - A).

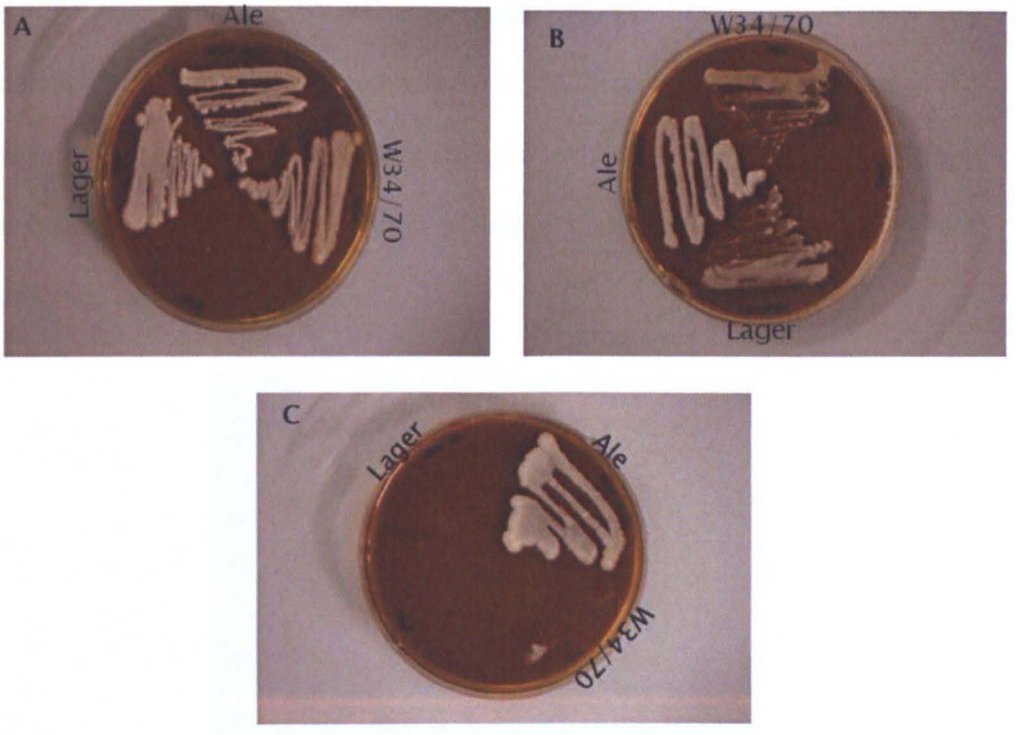



**Figure 3.1 - Streak pattern of production lager (W34/70) and control lager (NCYC1116) and ale (NCYC2593) strains on YPD agar plates after 5 days incubation at (A) 25 °C, (B) 34 °C and (C) 37 °C.**

### 3.2.2 Melibiose Utilisation

To assess the capacity of W34/70 to utilise melibiose, two methods were deployed: (1) the X-α-gal Assay (Watkins *et al.*, 1998) (section 2.3.2.1, chapter 2) and (2) growth on meliobiose (Dmochowska *et al.*, 1990)(Section 2.3.2.2, Chapter 2)

#### 3.2.2.1 X-α-gal Assay

Melibiose utilisation was assessed using the X-α-gal assay (Section 2.3.2.1, Chapter 2) incubated at 27°C for 60 min. It was observed that W34/70 was able to generate an insoluble blue-coloured product indicating the capacity to cleave X-α-gal utilise via α-galactosidase formation, whilst the NCYC 2593 ale yeast strain did not yield any such change in the solution's colour (Figure

3.2 ). Surprisingly the NCYC lager strain (NCYC 1116) did not show any colour change following incubation at 27°C for 60 mins (Figure 3.2 ). To ensure that this result was not a function of incubation time NCYC 1116 strain was incubated at 27°C for a longer period of 3 days. No change in colour was observed (data not shown).

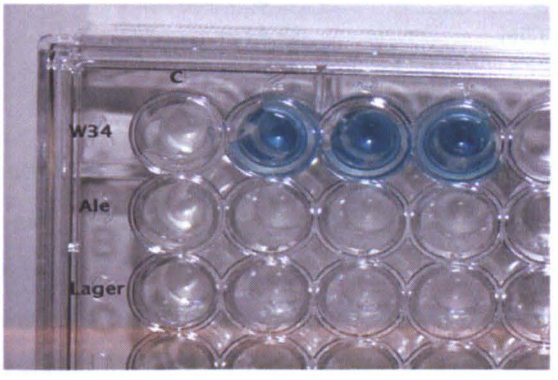



**Figure 3.2 - Incubation of W34/70, NCYC1116 lager and NCYC2593 ale yeast strains in X-α-gal solution at 27°C. Formation of a blue colour indicates substrate hydrolysis. "C" indicates reaction control containing sterile water instead of X-α-gal. Triplicate results are been shown.**

### 3.2.2.2 Melibiose Utilization Assay

To confirm the results observed in section 3.2.2.1, each strain was inoculated into growth media containing melibiose as the sole source of carbon and incubated for 48 hrs at 27 °C (Section 2.3.2.2, Chapter 2). Growth or utilisation of the melibiose was indicated by the change in colour of the medium from blue / green to yellow and by the accumulation of gas as a bubble in the Durham tube. W34/70 demonstrated the presence of gas in the inverted Durham tubes and a change of the solution's colour from green to yellow (Figure 3.3). In contrast no growth occurred in water (negative control) (Figure 3.3). Gas generation and colour change was not observed for the ale (NCYC 2593) and lager (NCYC 1116) (Figure 3.3) strains in melibiose.

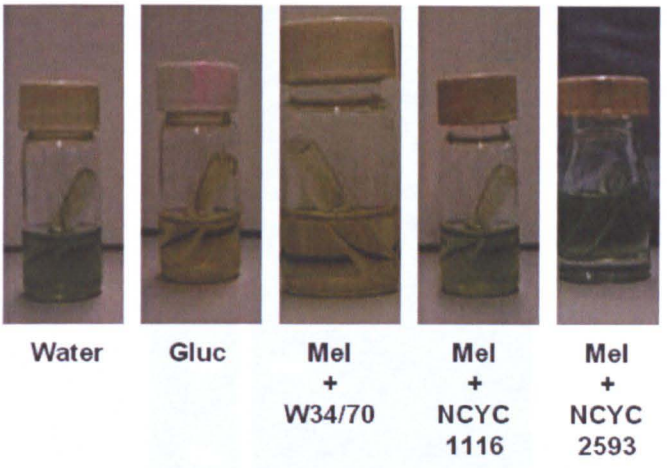



**Figure 3.3 - Utilisation of melibiose by the production lager (W34/70), control lager (NCY 1116) and control ale (NCYC 2593) strains. For each assessment a positive control containing Glucose (Gluc) as sole carbon source and a negative control in water was utilized. Carbon source utilisation is indicated by colour change and gas formation in the form of bubbles at the top of inverted durham tubes. Triplicate reactions were conducted.**

### 3.2.3 Differential Yeast Mitochondrial DNA

To confirm ale of lager genotype total cellular DNA was extracted, quantified and subjected to a novel PCR technique which amplifies ale or lager specific sequences using a polymerase chain reaction (PCR) (sections 2.3.3 and 2.3.4, Chapter 2). Figure 3.4 demonstrates the electrophoretic mobility of various amplicons on an agarose gel. Analogous with characteristic lager-type yeast, the production strains, W34/70, yielded a single PCR product of the correct size with regard to lager genotype; in contrast, both wild-type (S288C) and ale (NCYC 2593) yeasts exhibited a much smaller product.

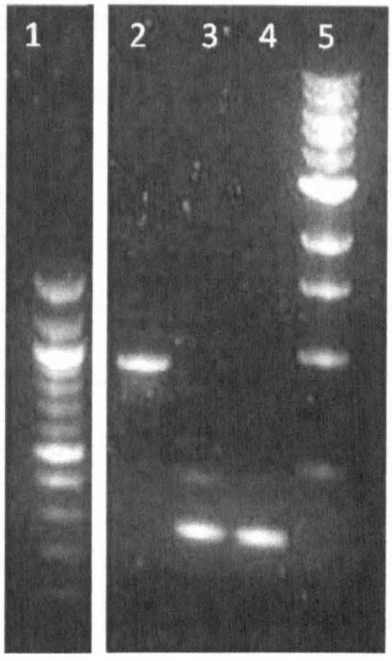



**Figure 3.4 - PCR differentiation of ale and lager strains. PCR conditions were those in Section 2.3.4 (Chapter 2). Lanes 1 and 5; 100bp and 1kb DNA ladder respectively (New England Biolabs, UK), lane 2; W34/70 (lager), lane 3; S288C (laboratory *S.cerevisiae* strain) and lane 4; NCYC 2593 (ale). Amplification products were run on 1% agarose gel and visualized using SyBr green dye (0.001%).**

### 3.2.4 Standardization of Cooling

In order to study the impact of cooling on brewing yeast it was necessary to establish a model laboratory working system that would permit the cooling of yeast suspensions and slurries in a manner that represented the process routinely conducted within a full scale brewery. Using mini fermentation vessels (Section 2.4.1, Chapter 2) and a project dedicated incubator the cooling efficiency of the incubator was examined by assessing the impact of incubation on the thermal profile of a yeast suspension in spent YPD ($1.5 \times 10^7$ cells / ml) under both stirred and non-stirred conditions (Section 2.6, Chapter 2).

Figure 3.5 demonstrates that the non-stirred cell suspensions reached the designated incubator temperature of 15°C after 2 hrs. The stirred FVs were substantially warmer than those not stirred (by about 1.3°C). This was primarily attributed to the heat generated due to the constant physical motion of the media as a consequence of continuous stirring. As a consequence all subsequent experiments were conducted using a suitable temperature correction (the incubator target temperature was set at 1.3°C lower than the desired temperature).

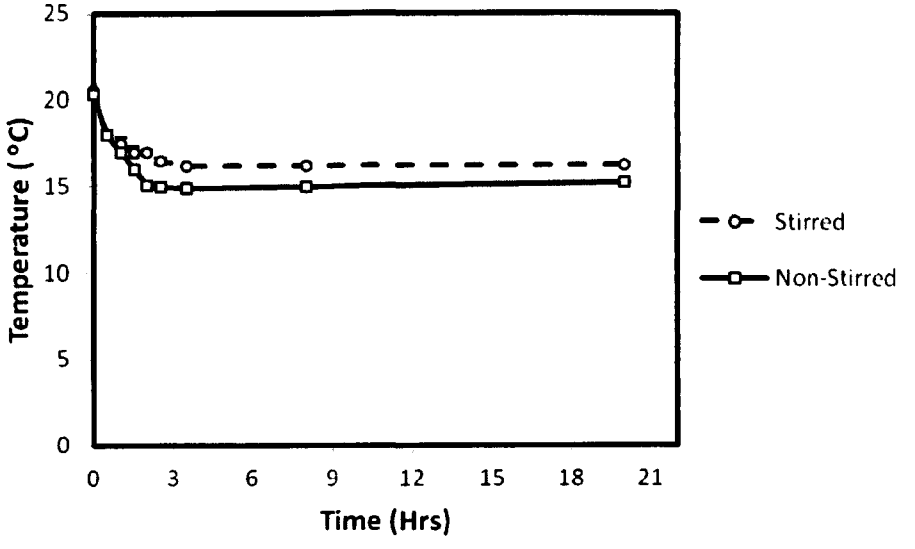



**Figure 3.5 - Thermal downshift profile of W34/70 brewing lager yeast at cell densities mimicking pitching rates (15 x $10^6$ cells / ml) in spent YPD. Where indicated cell suspensions were constantly agitated at 400 rpm using multi place magnetic stirrer (MPMS) plates. Data represents the mean of three replicates with error bars representing the standard deviation.**

Thermal downshift profiles using W34/70 yeast cell suspensions with a concentration of cells typically utilized for inoculation (pitching) into brewing fermentation vessels (Section 2.7, Chapter 2) are shown in Figure 3.6A. The specific temperature ranges (25°C to 15, 10 and 4°C) were chosen in an

attempt to mimic temperature ranges experienced by yeast strains during the course of fermentation and yeast handling. When subjected to different cooling temperatures the yeast suspension demonstrated different rates of temperature downshift. The time required for the yeast cell suspension to cool down from 25°C to 15°C, 10°C and 4°C was 120 mins on each ocassion (Figure 3.6A).

Thermal downshift profiles were also obtained for cell suspensions with higher yeast cell densities suspended in spent wort "beer" (Section 2.8, Chapter 2) to mimic the cell concentrations typically observed in yeast slurries stored between successive fermentations. The thermal downshift range of 15 to 10 °C was selected to imitate the temperature changes experienced by the yeast cells during latter stages of a typical brewing fermentation whilst ranges of 10 to 4 °C and 15 to 4 °C imitate the typical temperature reduction experienced by yeast crops at the end of fermentation cycle and on transfer to storage tank. Irrespective of the conditions applied, W34/70 yeast slurries (in "beer") attained the desired final temperatures after 60 min of uninterrupted cooling under continuous agitation (Figure 3.6B).

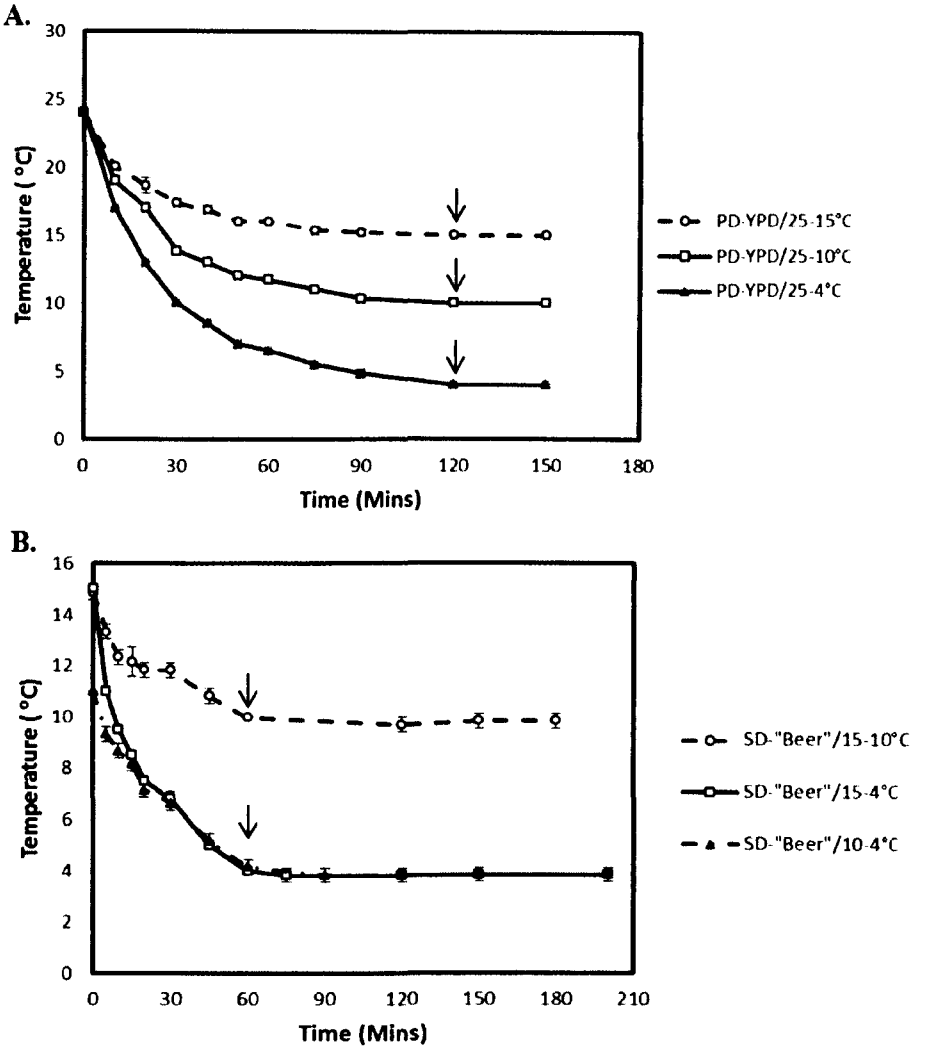



**Figure 3.6 - Thermal downshift profiles of W34/70 cultures mimicking different yeast handling conditions prevalent in the brewery. (A) Pitching Density (PD) - YPD indicates yeast suspended in spent YPD (1.5 x $10^7$ cells / ml). (B) Slurry Density (SD) -YPD represents yeast biomass (30% wet w/v) in spent wort "beer" suspension (7.6% abv). All cell suspensions were constantly agitated at 400 rpm using multi place magnetic stirrer plates. Data represents mean of three replicates with error bars representing the standard deviation.**

The impact of cell density on cooling times following a thermal downshift from 15 °C to 10 °C (Figure 3.7) suggested that cooling rates were similar for both pitching (1.5 x $10^7$ cells/ml) and slurry cell densities (30% w/v) in spent YPD "beer" resuspension media.

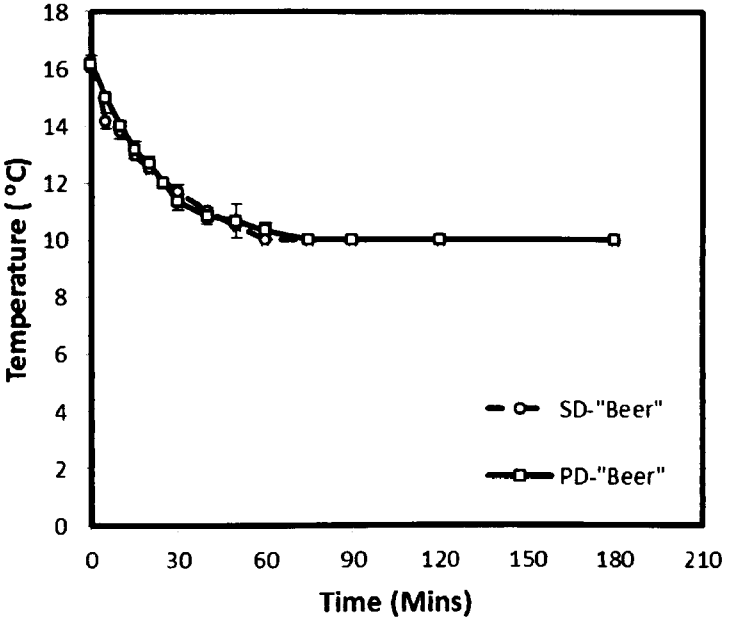



**Figure 3.7 - Thermal downshift profile of W34/70 brewing lager yeast at different cell densities following resuspension in spent wort "beer" (7.6% abv). PD indicates pitching densities (1.5 x$10^7$ cells / ml) whilst SD represents slurry densities (30% w/v). All cell suspensions were constantly agitated at 400 rpm using multiplace magnetic stirrer plates. Data represents the mean of three replicates with error bars representing standard deviation.**

## 3.3 Discussion

Brewery yeast comprises naturally selected strains of the genus *Saccharomyces* that have adapted to growth and fermentation in the complex media wort whilst experiencing various stress conditions. Ale and lager fermenting brewery strains differ from one another and also the standard laboratory strains in a number of ways including morphology, genetic composition, sporulation characteristics and perhaps most importantly their stress responses (Boulton and Quain, 2001) (Smart, 2007) (Tu *et al.*, 1996). To establish that the production strains W34/70 was indeed a lager strain it was deemed crucial to confirm lager phenotype.

### 3.3.1 Assessment of lager phenotype of W34/70

W34/70 was unable to grow at 37 °C (see Figure 3.1), a phenotype confirmed as "lager" by a similar growth inhibition of the control lager strain (NCYC 1116). In contrast the control ale strain (NCYC 2592) was able to grow at this temperature. Although the precise reason for this phenomenon is not known it has been established that all lager strains exhibit this phenotype (Wu *et al.*, 2001) (Sato *et al.*, 2002). It has been proposed that lager strains exhibit poor growth at higher temperatures and strong growth at lower temperature because of the contribution of the cryophilic *S.bayanus* to the lager yeast genome (Huang *et al.*, 1998) (Tehlivets *et al.*, 2007).

Lager strains may also be differentiated from ale strains by their capacity to utilize melibiose as a growth substrate; a phenotype not reported for any ale strain. Melibiose utilization is a well-known indicator of lager yeast phenotype (Boulton and Quain, 2001) and is primarily possible due to the presence of α-galactosidase encoding *MEL* gene(s) (Hwang *et al.*, 1989). Electrokaryotyping

analysis of five different *S.pastorianus* yeast strains demonstrated the presence of a single *MEL* gene in each of the investigative strains (Francois and Parrou, 2001). Subsequent *Saccharomyces pastorianus* W34/70 genome sequencing confirmed the presence of a single lager-brewing yeast specific melibiase-encoding gene (Nakao *et al.*, 2009) in the sub-telomeric region of the mosaic Sc-Sb chromosome X. The W34/70 yeast strain utilized in this project was able to cleave the chromogenic substrate X-α-gal (Figure 3.2) and also utilize melibiose as a growth substrate (Figure 3.3). Both these observations together suggest the presence of a functional α-galactosidase enzyme in the current W34/70 strain in accordance with previous phenotypic and genetic reports outlining lager yeast characteristics (Francois and Parrou, 2001) (Nakao *et al.*, 2009) (Boulton and Quain, 2001). The final confirmation of W34/70*'s* lager-brewing characteristic was derived from a PCR-based DNA amplification assay (Figure 3.4) which unequivocally confirmed the lager phenotype of the utilized W34/70 strain.

The inability of NCYC 1116 (used as a control lager strain) to degrade X-α-gal (Figure 3.2) or utilize melibiose as a single carbon source for growth (Figure 3.3) suggests that this particular lager strain lacks a functional α-glucosidase enzyme. This is probably due to the lack of, or truncation of, *MEL* gene(s) in the genome of NCYC1116 (National Collection of Yeast Cultures; http://www.ncyc.co.uk/yeast-ncyc-1116.html). The genome sequence for NCYC1116 is not available and therefore the precise reason for this discrepancy is not yet known and merits further work.

### 3.3.2 Development of Cooling Protocols

It was determined that cooling efficiency was dependent on the application, or otherwise of stirring. It was observed that cooling when agitation was deployed resulted in a higher end point temperature than when agitation was not deployed (Figure 3.5). It was therefore deemed necessary to adjust the temperature of the incubator (1.3°C lower than the actual temperature required) to achieve the desired low temperature when applying continuous stirring.

Cooling efficiency was dependent on the media in which cooling was applied but not on the extent of cooling required (although this was not extensively different). Similar cooling times (60 minutes) were observed following temperature downshift of yeast suspensions in "beer" from 15 °C to 10/4 °C and from 10 °C to 4 °C using laboratory-scale mini fermentation vessels (Figure 3.6B). Interestingly, no significant difference was observed between the cooling rates of the pitching and slurry cell densities ($p > 0.05$ using Student's T Test) suspended in spent wort "beer" (Figure 3.7). This suggests that for a laboratory-scale experiment, cooling rates were unaffected by the cell concentration as long as "beer" was utilized as the resuspension medium.

## 3.4 Conclusion

In this Chapter the lager phenotype of W34/70 was confirmed, however it was observed that the lager strain NCYC 1116 appears to lack the melibiose phenotype normally associated with lager strains. This will be further investigated in the Smart Laboratory but was not the subject of further study in this thesis. A suitable model working system was established in which cooling rates could be measured effectively. This approach was utilised to investigate the impact of cooling on the physiology of the lager strain W34/70 in Chapter 4.

# CHAPTER 4: IMPACT OF STORAGE ON FRESHLY PROPAGATED YEAST SLURRY

## 4.1 Introduction

Recovery or "cropping" of yeast biomass, achieved manually or through automated systems, at the completion of fermentation is widely employed in the brewing industry. Although, yeast crops might be used directly for inoculating (pitching) another fermentation, the more usual approach is pumping the cropped yeast slurry into attmeperated storage vessels until required for subsequent use. Maintenance of yeast quality during storage in terms of microbial stability (Boulton and Quain, 2001), viability (McCaig and Bendiak, 1985b), cellular physiological state (Pickerell *et al.*, 1991), membrane function (Heggart *et al.*, 1999) and key fermentation attributes (Heggart *et al.*, 1999) (Pickerell *et al.*, 1991) (Rhymes and Smart, 2001) is vital for optimal yeast performance during fermentation. To achieve this, cropped yeast is usually stored anaerobically under diluted spent wort "beer" at low temperatures (Boulton and Quain, 2001) before subsequent re-pitching.

Brewing yeast quality during storage can be influenced by a number of factors including the duration of storage (McCaig and Bendiak, 1985a) (McCaig and Bendiak, 1985b), application of oxygenation (Verbelen *et al.*, 2009b) and agitation (McCaig and Bendiak, 1985a), $CO_2$, nutrient availability (Smart *et al.*, 1999) and temperature (McCaig and Bendiak, 1985b). Typically, cropped yeast biomass is uniformly cooled (generally to 3-4°C) by passage through a chiller (Gibson *et al.*, 2007) and is maintained at these temperatures with periodic agitation (to achieve slurry homogeneity) under spent wort

"beer". Non-optimal storage temperatures can result in impaired fermentations thus causing disruption in supply chain efficiency and escalation of production costs.

The impact of thermal downshift on brewing yeast has not been the subject of extensive investigation. This is surprising in light of the routine application of low temperature storage to brewing yeast during industrial handling. It has been proposed that tolerance to cold stress may be strain dependent but that lager yeast are not necessarily more tolerant that ale strains (Leclaire and Smart, in preparation). Having established a model working system and conducted assessment of the cooling times of *Saccharomyces pastorianus W34/70* slurries previously (Chapter 3), this chapter focuses on the impact of different storage temperatures on cell viability (Section 4.2.1) and indicators of yeast physiological state. Yeast physiological status was gauged by assessing the (i) cellular proton efflux using the acidification power test (Section 4.2.2), (ii) intracellular glycogen and (iii) trehalose levels (Section 4.2.3), and (iv) intracellular fatty acid distribution (Section 4.2.4).

## 4.2 Results

### 4.2.1 Impact of low temperature storage on cell viability

Viability is a key performance indicator of yeast storage between successive fermentations. It is proposed that modification in storage parameters applied would therefore require validation using viability assessment. To address this the viability of *W34/70* populations stored at 25°C, 10°C and 4°C was assessed using dyes that determine the intracellular reductive capacity (citrate methylene violet, CMV; Section 2.11.1, Chapter 2) or maintenance of trans-membrane potential (oxanol; Section 2.11.2, Chapter 2).

Where CMV had been applied to assess viability, it was observed that the duration and temperature at which the lager strain was stored was important. A marked decline in the capacity of freshly propagated yeast cells to reduce CMV was observed irrespective of storage temperature (Figure 4.1); the extent to which viability was impaired was, however, a function of the temperature applied. Surprisingly the viability of yeast stored at 4°C was not higher than that observed at 10 °C. In line with expectation though yeast stored at 25 °C exhibited a statistically significant reduction in viability after 24 and 48 hrs ($p < 0.05$) when compared to populations stored for a similar duration at both 4 and 10°C (Figure 4.1).

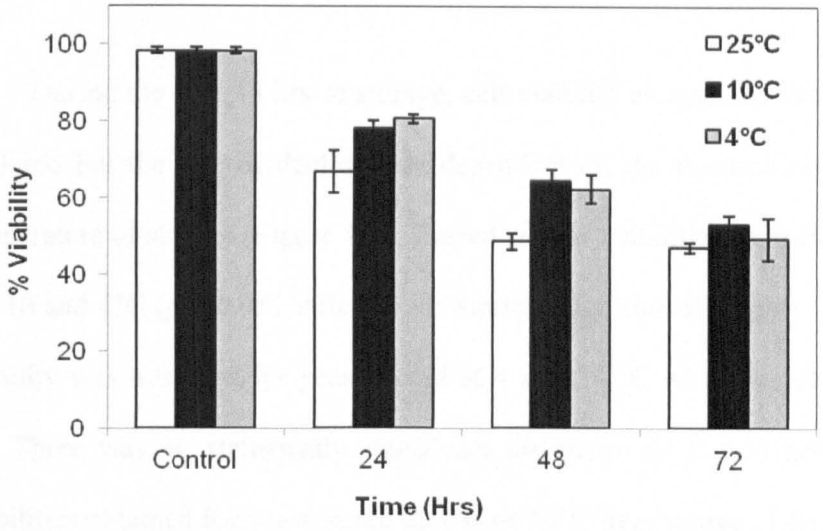



**Figure 4.1 - Percentage viability of *W34/70* lager yeast slurry (1 x $10^9$ cells / ml) in sterile water during anaerobic storage at 25 °C, 10 °C and 4 °C under continuous agitation (400 rpm using multiplace magnetic stirrer plates). Viability was determined using Citrate Methylene Violet (CMV). Data represents mean of three replicates. The error bars depict the standard deviation.**

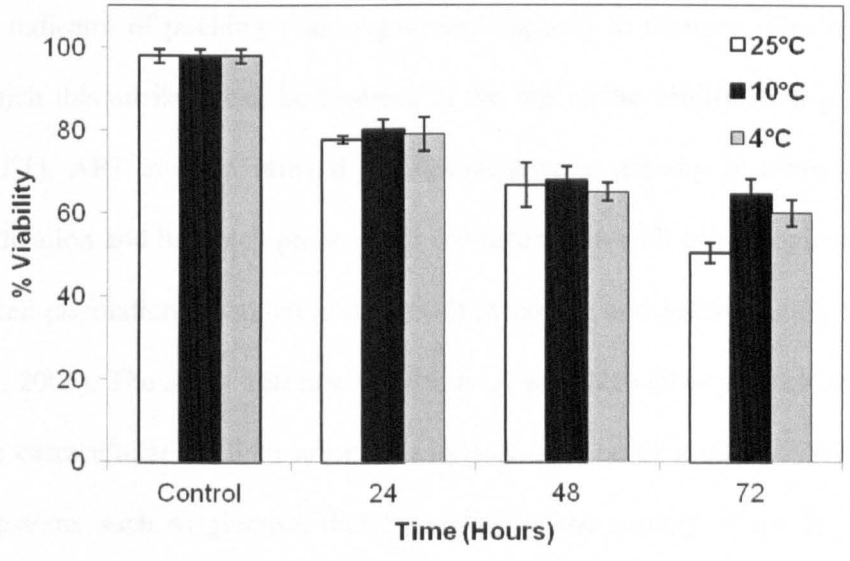



**Figure 4.2 - Percentage viability of *W34/70* lager yeast slurry (1 x $10^9$ cells / ml) in sterile water during anaerobic storage at 25 °C, 10 °C and 4 °C under continuous agitation (400 rpm using multiplace magnetic stirrer plates). Viability was determined using Oxanol [bis-(1,3-dibarbituric acid)-trimethine oxanol; DiBAC4]. Data represents mean of three replicates. The error bars depict the standard deviation.**

During the first 48 hrs of storage, cell viability as assessed using oxanol declined but the rate of decline was dependent on the duration and not the temperature of storage (Figure 4.2). Indeed similar viabilities were observed at 25, 10 and 4°C ($p > 0.05$). After 72 hrs storage, significantly higher ($p < 0.05$) viability was observed for yeast stored at 4 and 10 °C when compared to 25 °C. There was no statistically significant difference ($p < 0.5$) between the viabilities obtained for yeast stored at 4°C or 10°C irrespective of the duration of storage applied.

### 4.2.2 Impact of low temperature storage on cellular proton efflux

Following storage, yeast slurries are inoculated (pitched) into wort in order to initiate fermentation. The rapid uptake of sugars from the wort is considered an indicator of pitching yeast vigour and capacity to ferment. One means by which this attribute can be assessed is the use of the acidification power test (APT). APT is often utilized for assessing yeast activity in terms of sugar utilization and has been proposed to indicate the overall physiological state of a cell population (Mathieu *et al.*, 1991) (Siddique and Smart, 2000) (Sigler *et al.*, 2006). The assay assesses the ability of a viable cell population to acidify the extracellular medium prior to- and post-addition of a metabolizable sugar substrate, such as glucose, thereby reflecting the activity of the $H^+$ ATPase located in the cell membrane. When suspended in water, yeast cells passively extrude protons ($H^+$) into the outer medium in order to equilibrate with the medium's pH (Mathieu *et al.*, 1991). This property is measured by Water Acidification Power (WAP) and has been suggested to correlate directly with cell's endogenous energy reserves (Mathieu *et al.*, 1991) (Siddique and Smart,

2000). Following addition of a sugar substrate, like glucose, the reduction in the pH of the cell suspension is measured by GAP which represents the overall glycolytic activity of the cell upon sugar addition (Sigler *et al.*, 1981). The Glucose Induced Proton Efflux (GIPE) is calculated by subtracting WAP from GAP and it reflects the net proton efflux exclusively due to sugar addition (Siddique and Smart, 2000).

In the current work the Acidification Power Test (APT) (Section 2.12, Chapter 2) was used to determine the proton efflux for yeast freshly propagated (control) and post storage slurries which had been incubated at 25°C, 10°C and 4°C for up to 72 hours. Figure 4.3A demonstrates the WAP profiles of each slurry sample assessed. WAP was surprisingly absent in freshly propagated slurries. With progression of storage a gradual decrease in WAP levels were observed for slurries stored at 25°C and 10°C, between 24 to 72 hrs. Slurry stored at 4°C demonstrated lower WAP potential after 24 hrs ($p < 0.05$) and a significant increase in this attribute after 72 hrs of incubation (Figure 4.3A) ($p < 0.05$). GAP in stored yeast was slightly higher than the corresponding freshly propagated control at all temperatures investigated (Figure 4.3B). *W34/70* slurries stored at both 10°C and 4°C appeared to have similar GAP levels throughout the duration of storage. GAP potential at 25°C was higher than cold-stored slurries after 24 hrs ($p < 0.05$) but this trend was not maintained after 48 or 72 hrs. An analysis of GIPE (proton efflux resulting only from glucose utilisation) (Figure 4.3C) demonstrated higher ($p < 0.05$) exogenous sugar utilization capacity for *W34/70* maintained at 10°C in comparison to 4°C after 72 hours of storage.

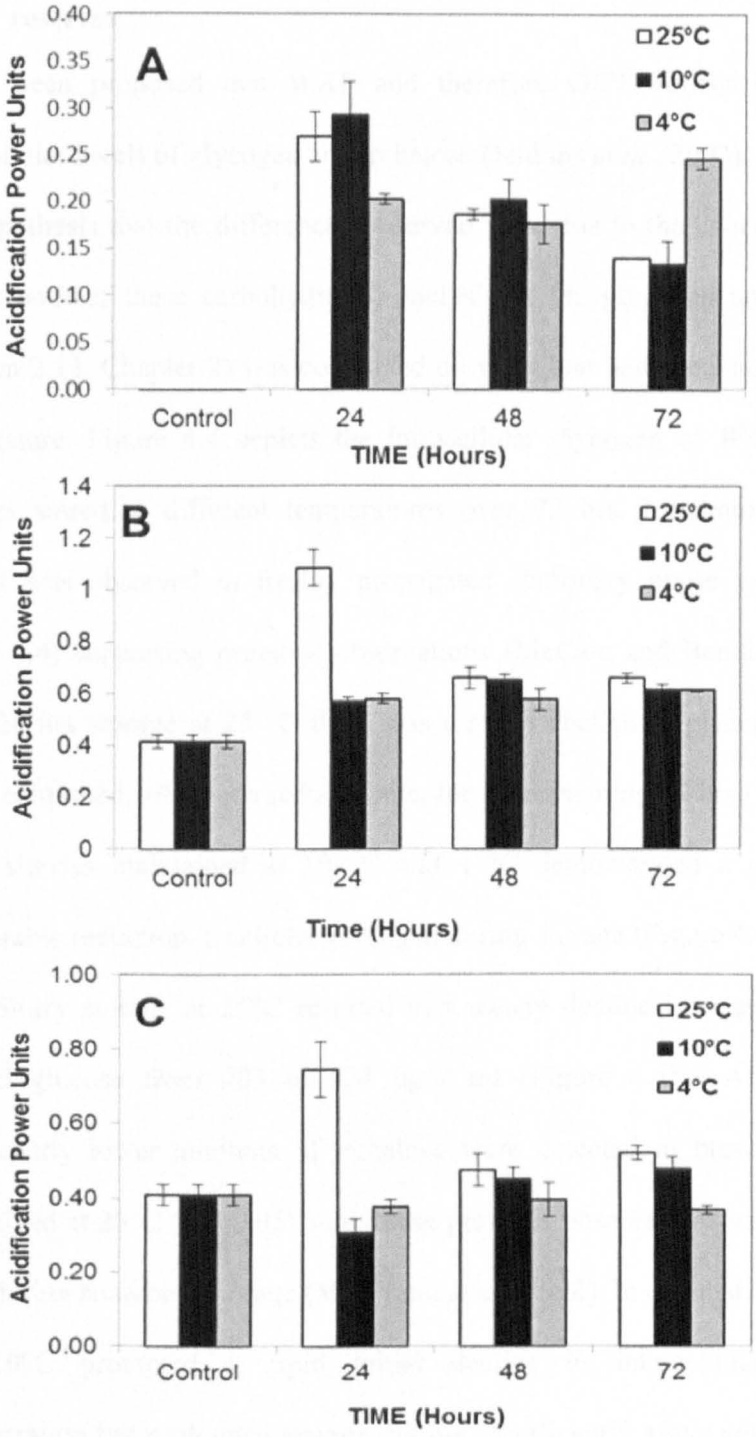



**Figure 4.3 - Water Acidification Power (WAP; A), Glucose Acidification Power (GAP; B) and Glucose-Induced Proton Efflux (GIPE; C) of *W34/70* lager yeast slurry aliquots (1 x $10^9$ cells/ml) during 72 hours of anaerobic storage at 25°C, 10°C and 4°C under continuous agitation (400 rpm using multiplace magnetic stirrer plates). Data represents mean of three replicates. The error bars depict the standard deviation.**

### 4.2.3 Impact of low temperature storage on intracellular carbohydrate reserves

It has been proposed that WAP and therefore GIPE reflect in part the intracellular levels of glycogen and trehalose (Jenkins *et al.*, 2003). To address the hypothesis that the differences observed were due to the concentration of one or both of these carbohydrates, analysis of the glycogen and trehalose (Section 2.13, Chapter 2) was conducted on yeast that had been stored at each temperature. Figure 4.4 depicts the intracellular glycogen of *W34/70* slurry samples stored at different temperatures over 72 hrs. Maximum glycogen content was observed in freshly propagated stationary phase yeast (0 hrs, Figure 4.4) supporting previous observations (McCaig and Bendiak, 1985b). After 24 hrs storage at 25 °C there was a sharp decline in glycogen content which continued, albeit at a reduced rate, for the remaining 72 hrs (Figure 4.4). Yeast slurries maintained at 10 °C and 4 °C demonstrated a gradual and comparable reduction in cellular glycogen during storage (Figure 4.4).

Slurry storage at 25°C resulted in a steady decline in yeast trehalose-derived glucose from 203 to 124 μg / ml (Figure 4.5). After 72 hrs, significantly lower amounts of trehalose were detected in brewing slurries maintained at 25°C ($p < 0.05$) supporting previous observations with distilling yeast during anaerobic storage (Morimura *et al.*, 1998). In contrast, storage at 4 and 10°C prompted a rapid initial decline in intracellular trehalose concentration but prolonged storage did not significantly affect concentrations of this disaccharide (Figure 4.5).

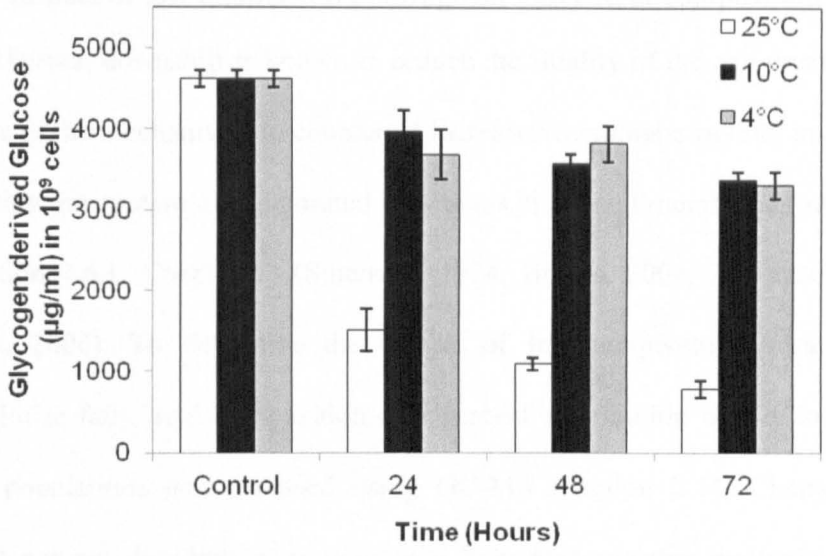



**Figure 4.4 - Intracellular glycogen content in *W34/70* cell populations (1 x $10^9$ cells) stored anaerobically at 25 °C, 10 °C and 4 °C for 72 hours with continuous agitation (400 rpm using multiplace magnetic stirrer plates). Data represents mean of three replicates. The error bars depict the standard deviation.**

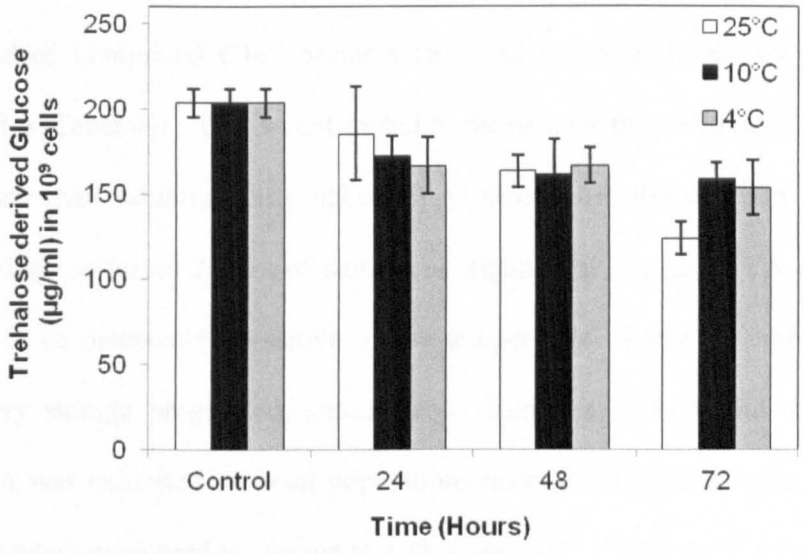



**Figure 4.5 - Intracellular trehalose content in *W34/70* cell populations (1 x $10^9$ cells) stored anaerobically at 25 °C, 10 °C and 4 °C for 72 hours with continuous agitation (400 rpm using multiplace magnetic stirrer plates). Data represents mean of three replicates. The error bars depict the standard deviation.**

### 4.2.4 Impact of low temperature storage on Fatty Acid composition

Thermal downshift is known to reduce the fluidity of the cell membrane and one of the mechanisms to counteract increased membrane rigidity involves enhancing the content of unsaturated fatty acids in the cell membrane (outlined in Section 1.6.1, Chapter 1) (Sinensky, 1974, Beales, 2004, Al-Fageeh and Smales, 2006). To determine the impact of low temperature storage on intracellular fatty acid composition, the percent distribution of FA in yeast slurry populations was assessed using GC-MS (Section 2.14, Chapter 2). Overall percent distribution of individual fatty acid moieties suggested that palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0) and oleic acid (18:1) were the most abundant, whilst the remaining fatty acids were present in minute quantities (overall less than 5%). Thus, for further assessments, analysis was restricted to the major FAs (Table 4-1).

In freshly propagated *W34/70* (control), the predominant fatty acid residues comprised C16 species C16:1 and C16:0 followed by C18:1 and C18:0 (Table 4-1). UFAs contributed to the cellular fatty acid pool to a greater extent than saturated fatty acids (SFA) throughout the duration of storage. During the initial 24 hrs of storage no significant change in FA distribution could be detected irrespective of the temperature of storage employed. As slurry storage progressed, simultaneous increases in SFA and decreases in UFA was exhibited by yeast populations maintained at 25°C (after 48 and 72 hrs) when compared to storage at both 4 and 10°C (Table 4-1). As a result, the cell membranes of brewing yeast populations exposed to thermal downshift demonstrated higher unsaturation than those maintained at 25°C after 48 and 72 hrs of storage. Notably, there was no difference ($p > 0.05$) in the degree of

membrane unsaturation between slurries stored at 4 and 10°C throughout storage (Table 4-1). Interestingly, a slight adjustment in Unsaturation Index (UFA/SFA denoted UI) was observed between control and cold-stored brewing yeast slurries (from 1.39 to 1.42;Table 4-1) after 72 hrs of storage.

**Table 4-1 - Fatty Acid distribution (%) in *W34/70* cell populations (1 x $10^9$ cells) stored anaerobically at 25 °C, 10 °C and 4 °C for 72 hours with continuous agitation (400 rpm using multiplace magnetic stirrer plates). Data is represented as percentage of the total fatty acid and has been shown as mean ± standard deviation of three independent replicates.**

| Fatty Acid | Control | 24 (Hours) | | | 48 (Hours) | | | 72 (Hours) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 25.°C | 10°C | 4°C | 25°C | 10°C | 4°C | 25°C | 10°C | 4°C |
| FA – 16:0 | 31 ± 0.04 | 31 ± 0.35 | 30 ± 0.1 | 30 ± 0.57 | 31 ± 0.34* | 30 ± 0.09 | 30 ± 0.25 | 30 ± 0.35 | 30 ± 0.10 | 30 ± 0. |
| FA - 16:1 | 38 ± 0.02 | 38 ± 0.37 | 39 ± 0.55 | 38 ± 0.76 | 38 ± 0.56 | 38 ± 0.32 | 38 ± 0.13 | 37 ± 0.17 | 38 ± 0.24 | 38 ± 0. |
| FA - 18:0 | 11 ± 0.22 | 12 ± 0.12 | 11 ± 0.24 | 11 ± 0.32 | 12 ± 0.21* | 11 ± 0.13 | 11 ± 0.23 | 12 ± 0.22 | 11 ± 0.07 | 11 ± 0. |
| FA - 18:1 | 21 ± 0.26 | 20 ± 0.11 | 20 ± 0.25 | 20 ± 0.27 | 20 ± 0.21 | 20 ± 0.10 | 20 ± 0.18 | 21 ± 0.30 | 21 ± 0.31 | 20 ± 0. |
| SFA | 42 ± 0.25 | 42 ± 0.47 | 41 ± 0.30 | 42 ± 0.81 | 43 ± 0.55* | 42 ± 0.21 | 42 ± 0.31 | 42 ± 0.13 | 41 ± 0.15 | 41 ± 0. |
| UFA | 58 ± 0.25 | 58 ± 0.47 | 59 ± 0.30 | 58 ± 0.81 | 57 ± 0.55* | 58 ± 0.21 | 58 ± 0.31 | 58 ± 0.13 | 59 ± 0.15 | 59 ± 0. |
| UI | 1.39 | 1.36 | 1.44 | 1.4 | 1.33* | 1.4 | 1.39 | 1.37* | 1.42 | 1.42 |

*** indicates significant difference ($p < 0.05$) between means analyzed using one way ANOVA with Stukey-HSD post hoc correction. C16:0 - palmitic acid; C16:1 - palmitoleic acid; C18:0 - stearic acid; C18:1 - oleic acid;**

**SFA - Saturated Fatty Acid (C16:0 + C18:0); UFA – Unsaturated Fatty Acid (C16:1 + C18:1); UI – Unsaturation Index (UFA/SFA)**

## 4.3 Discussion

### 4.3.1 Impact of low temperature storage on yeast viability

The primary criterion for distinguishing between viable and non-viable cells in a microbial population is the ability of the former to undergo active cell division. Although, classical microbiological methods using cultivation-based techniques might be more suited for direct estimation of cellular growth potential, their inability to produce quick results has propelled the use of the so called "vital" stains for assessing yeast cellular viability (Boulton and Quain, 2001). Perhaps the most widely used stain for brewing yeast's viability assessment is methylene blue. However, methylene blue has a propensity to over-estimate viabilities, a shortcoming which becomes increasingly pronounced with drop in culture viability (less than 90%) (Heinisch *et al.*, 1998) (Smart *et al.*, 1999).

Citrate Methylene Violet (CMV) has been shown to be capable of unequivocal differentiation between viable and stress-induced non-viable cells (Smart *et al.*, 1999). Although the exact mechanism by which CMV determines cellular viability is not very well understood, recent reports on the mode of action of methylene blue (May *et al.*, 2003) (Shi *et al.*, 2011) provide important insights since both dyes belong to the same class. In cultured pulmonary endothelial cells, transmembrane reductases reduce methylene blue into a colourless, lipophilic form which diffuses inside the cell and can be re-oxidised before being sequestered within the cells (Merker *et al.*, 1997). Thus, yeast populations which retain the capacity to reduce CMV to its colourless form are deemed viable and cells which have lost the potential to do so are considered non-viable (Smart *et al.*, 1999). Oxonols are lipophilic, anionic

dyes which are sensitive to cell membrane potential (Epps *et al.*, 1994) and have been demonstrated as a viability marker for brewing yeast (Boyd *et al.*, 2003). Eukaryotic cells typically maintain an electrochemical potential gradient across their cytoplasmic membrane. When the yeast cell membrane is damaged, this electrochemical gradient is compromised and cell trans-membrane potential is reduced (membranes become depolarised) (Zhang and Crow, 2001). Oxonols enter depolarised cells and accumulate intracellularly by binding to hydrophobic sites on proteins and disrupted membranes (Epps *et al.*, 1994) and as a result can provide an indication of cell viability (Mason *et al.*, 1995). Viable yeast cells demonstrate no fluorescence whilst non-viable cells demonstrate a concomitant increase in fluorescence (Boyd *et al.*, 2003) (Epps *et al.*, 1994).

In this chapter, exposure of yeast slurries to thermal downshift negatively affected cellular viability and the deterioration in population viability progressed as a function of storage duration. Preservation of W34/70 populations, in terms of their ability to reduce CMV (Figure 4.1) and maintenance of cell membrane potential (Figure 4.2), was not enhanced following the utilization of storage temperatures typically applied by the brewing industry (at 4 °C). This is supported by previous observations in an ale yeast (McCaig and Bendiak, 1985b). Surprisingly, 72 hrs of anaerobic storage caused more than a 40% loss in cellular viability even at 4°C (Figure 4.1 and Figure 4.2). Substantially higher viabilities have been previously reported during production scale yeast slurry storage (McCaig and Bendiak, 1985b). One reason for the observed discrepancy could be the use of constant agitation during storage which was applied in the current study to prevent thermal

gradients. Reduction in viability of agitated yeast slurries has been demonstrated to exceed that observed with non-agitated or periodically-agitated stored slurries (McCaig and Bendiak, 1985a).

### 4.3.2 Assessment of yeast physiology through proton efflux

Although viability tests are able to differentiate between live and dead fractions in a given microbial population, they do not provide any information regarding the physiological status of the viable yeast population. This may, to a limited extent, be accomplished by the application of the acidification power test (APT).

The absence of water acidification power (WAP) in aerobically cultivated yeast slurry (Figure 4.3A) was rather surprising since intracellular carbohydrate levels (especially glycogen) have been suggested to accumulate in yeast cells that have reached stationary phase in growth medium (Boulton and Quain, 2001) (Gabriel *et al.*, 2008). The drop in spontaneous acidification following storage at 25 and 10 °C was consistent with the hypothesis that endogenous energy reserves decrease in the nutrient deficient environment experienced during storage (McCaig and Bendiak, 1985b) (Sall *et al.*, 1988).

Following addition of a sugar substrate, the Glucose Acidification Power (GAP) represents the overall glycolytic activity of the population (Sigler *et al.*, 1981) and has been suggested to indicate the level of yeast metabolism (Sigler *et al.*, 1981) (Sigler *et al.*, 2006). Equivalent glucose-induced acidification at 10 and 4 °C for 72 hrs of slurry storage (Figure 4.3B) would thus suggest maintenance of similar glycolytic flux (and membrane state) at the higher storage temperature (10 °C) when compared with the more traditional storage approach (4 °C). GAP does not differentiate between endogenous and

exogenous carbon source utilisation leading to a proton efflux (Siddique and Smart, 2000) and thus could be considered the closest representation of carbon utilisation of yeast pitched (inoculated) into fresh wort. An increased GAP in this scenario is likely to reflect an accelerated capacity for assimilation of exogenous carbohydrate by *W34/70* cells stored at 25 °C for 24 hours (McCaig and Bendiak, 1985b) (Smart *et al.*, 1999). However sugar uptake is not the only attribute required of yeast during fermentation. Storage at high temperatures could be detrimental to cell viability and inoculating fermentations with excess dead cells can lead to impaired beer clarity (Finn and Stewart, 2002) and off-flavours (Pickerell *et al.*, 1991).

Glucose Induced Proton Efflux (GIPE) reflects the net efflux of protons across the cell membrane induced by the addition of the extracellular substrate (Siddique and Smart, 2000), glucose in the current study. The higher GIPE observed following 72 hrs of W34/70 incubation at 10 °C (in comparison to 4 °C) can probably be explained by a compensation effect. *W34/70* populations stored at 10 °C and exhibiting a poor WAP, potentially due to reduced intracellular carbohydrate reserves, may maintain GAP by increased exogenous carbon source utilisation which in turn enhances GIPE. If fermentation potential was dependent on wort sugar uptake alone, and assuming proton efflux is an effective measure of this, yeast populations stored at 10 °C could be considered to be more "fermentation ready" after 72 hours of storage than populations stored at 4 °C.

### 4.3.3 Assessment of yeast physiology through glycogen content

Intracellular levels of the major yeast storage carbohydrate glycogen have been regarded as an indicator of effective cropped brewing yeast slurry storage (Boulton and Quain, 2001) (Quain and Tubb, 1982). Yeast glycogen stores serve as a source of energy for maintaining cellular function during storage of cropped yeast and may be crucial for cell survival (Heggart *et al.*, 1999) (Murata *et al.*, 2006). Moreover yeast glycogen has been suggested to indicate the fermentation potential of stored yeast following pitching (Pickerell *et al.*, 1991) (Quain and Tubb, 1982). Indeed, yeast pitched with depleted glycogen reserves has been associated with lower cell viability, extended lag phase following pitching, reduced amino acid uptake and increased end-of-fermentation levels of diacetyl, acetaldehyde and sulphur dioxide (Pickerell *et al.*, 1991). A downshift in temperature activates the formation of glycogen as a physiological consequence of the activation of the General Stress Response (Gasch *et al.*, 2000) (Ruis and Schuller, 1995) (Schade *et al.*, 2004). Glycogen recycling occurs following exposure to heat shock and oxidative stress but has not been demonstrated to occur during exposure to cold temperatures. Instead there appears to be a significant accumulation of the polysaccharide (Aguilera *et al.*, 2007) (Schade *et al.*, 2004).

Acute degradation of glycogen was observed in the first 24 hrs of storage at 25 °C and the downward trend continued until 72 hrs (see Figure 4.4); final levels reached 17% of that exhibited by freshly propagated stationary-phased yeast. This observation supports the previous observations for an ale yeast at the same temperature (McCaig and Bendiak, 1985b). The comparable glycogen reduction in yeast slurries maintained at 10 and 4 °C does not support

the observations of McCaig and Bendiak (McCaig and Bendiak, 1985b) who reported an increased rate of glycogen reduction with increase in storage temperature from 5 °C to 10 °C. One possible reason for this difference may be that ale (which was the focus of the previous study) and lager (which is the focus of the current study) yeast strains respond in a dissimilar manner.

#### 4.3.3.1 Correlation between WAP and intracellular glycogen

Passive proton efflux (WAP) has been proposed to be directly influenced by the cellular glycogen content (Mathieu *et al.*, 1991). Using Pearson's correlation no significant association between passive proton efflux (WAP) and intracellular glycogen levels could be determined in the current study. Similar observations have been reported by Siddique and Smart (2000) for lager, ale and cider strains. However for yeast recovered from a full scale fermenter a positive correlation between WAP and intracellular glycogen levels was noted in a lager strain (Jenkins *et al.*, 2003), but these authors did not then examine the impact of storage on this relationship. One possible reason for the lack of correlation in certain circumstances concerns the fact that glycogen is not the only intracellular carbohydrate accumulated during stress and the General Stress Response also regulates the accumulation of trehalose (Gasch *et al.*, 2000). Trehalose utilisation could potentially contribute to WAP and thus the levels of this intracellular carbohydrate during storage were also assessed.

### 4.3.4 Assessment of yeast physiology through trehalose content

Trehalose, a non-reducing disaccharide, is primarily regarded as a stress protectant in yeast. Its role in providing stress resistance against a plethora of adverse environmental conditions including heat shock (Hottiger *et al.*, 1987),

osmotic (Hounsa *et al.*, 1998) and oxidative (Parrou *et al.*, 1997) stress, ethanol toxicity (Odumeru *et al.*, 1993), dessication (Eleutherio *et al.*, 1993) and nutrient starvation (Lillie and Pringle, 1980) is well documented. In *S.cerevisiae*, trehalose accumulation has been associated with maintaining cell viability at 10°C, 4°C and is absolutely critical for survival at 0°C, independent of active cellular growth (Aguilera *et al.*, 2007). Although the importance of trehalose in surviving low temperatures and freezing stress has been demonstrated in yeast (Kandror *et al.*, 2004), the exact functions of trehalose or multiple mechanisms governing its accumulation following cold shock have not yet been elucidated. Higher trehalose levels in pitching yeast have been reported to coincide with cell viability maintenance during the initial stages of fermentation thus prompting enhanced utilization of major carbohydrates (Guldfeldt and Arneborg, 1998).

Intracellular trehalose levels in yeast have been proposed to be an indicator of the amount of stress being levied upon a cell population on account of the sub-optimal environmental conditions (Majara *et al.*, 1996). The presence of similar amounts of trehalose following W34/70 slurry storage at 10 and 4 °C might thus indicate equivalent magnitudes of stress being generated in the two conditions. A significant positive correlation was observed between trehalose content and cell viability assessed by CMV ($r = 0.51$; $p < 0.01$) and oxonol ($r = 0.62$; $p < 0.01$) supporting previous observations (Damore *et al.*, 1991). Lille and Pringle (Lillie and Pringle, 1980) suggested that yeast accumulates trehalose under carbon starvation but this was not observed under storage conditions utilised in the current study which differed with respect to nutrient and gaseous environment and temperature conditions deployed by the

authors. It is proposed that yeast stored at 25 °C may need to utilize trehalose reserves as a source of carbon for synthesis and energy. No ATP generation takes place during trehalose dissimilation into glucose (Boulton and Quain, 2001) but clearly ATP is released during glucose utilisation even in anaerobic conditions. Furthermore it is proposed that the comparatively higher glycogen levels observed during storage at 10 and 4 °C might enable preservation of trehalose levels permitting the cell to deploy this disaccharide in the protection of cellular membranes during exposure to cold. In support of this hypothesis, Sall *et al.* (Sall *et al.*, 1988) reported little overall change in trehalose levels in yeast stored under brewing conditions (< 4 °C).

Trehalose (Guldfeldt and Arneborg, 1998) and glycogen (Quain and Tubb, 1982) content of pitching yeast has been suggested to predict yeast performance in subsequent fermentation. Using this as a criterion for yeast quality, it is proposed that storage at 10 °C and 4 °C results in yeast populations with similar intracellular levels of trehalose and glycogen.

### 4.3.5 Impact of low temperature storage on membrane fluidity

Exposure to low temperatures has considerable impact on plasma membrane lipid composition and fluidity (Russell, 1997). Normally the yeast cell membrane is quite flexible and present in a liquid crystalline form (Thieringer *et al.*, 1998). Following exposure to lower temperatures, the membrane structure becomes more ordered leading to a reduction in flexibility and conversion from the liquid-crystalline form to a more rigid gel phase state (Thieringer *et al.*, 1998). This reduced membrane fluidity results in impaired membrane function including slower diffusion rates and perturbed cross membrane protein and metabolite transport (Al-Fageeh and Smales, 2006).

Yeast overcomes this through homeoviscous adaptation (Sinensky, 1974) which, depending upon the organism and existent environmental conditions, might involve increasing the extent of membrane unsaturation (by elevated unsaturated fatty acid (UFA) formation), incorporation of cis-double bonds in existing lipids, higher methyl branching or shortening of fatty acid (FA) chains (Russell, 1997).

The lack of a significant difference between the unsaturation index (UI) measured in 10 °C- and 4 °C-stored W34/70 cell populations could possibly be explained by the gaseous environment employed during storage. Under hypoxic or anaerobic conditions yeast cells cannot actively generate unsaturated fatty acids (UFAs) because to do so, requires molecular oxygen for the desaturation reaction by Ole1p. Encoded by *OLE1*, this protein is the only known fatty acid desaturase in *S.cerevisiae* and catalyzes the formation of a double bond between carbons 9 and 10 of saturated fatty acid to generated corresponding unsaturated fatty acids (Zhang *et al.*, 1999). In the absence of a fresh UFA supply, it would be advantageous for cold-stored brewing yeast populations to conserve UFAs.

## 4.4 Conclusion

The rationale of yeast storage is to maintain yeast quality between successive fermentations and assure potential to perform. Whilst it is not surprising that storage at 25 °C leads to poor viability and a decline in physiological state, it appears to also result in an increased apparent capacity to utilise exogenous sugars, probably as a consequence of enhanced starvation. Although best brewing practise tends to favour the utilisation of 3-4°C during storage (Boulton and Quain, 2001) (Heggart *et al.*, 1999) for the conservation of yeast viability and physiological state it is proposed that for some strains, storage at 10 °C may be acceptable. Furthermore for some strains, including *W34/70*, storage at 10 °C may provide benefits. Indeed GIPE, an indicator of initial sugar uptake during fermentation, was higher for populations stored at 10°C. It is therefore proposed that the slurry storage temperature should be tailored to the yeast strain used. Where higher storage temperatures are applied there are obvious benefits in the reduction of energy inputs for slurry storage, providing these do not cause detrimental effects to the yeast and subsequent fermentation performance.

The use of propagated W34/70 yeast slurry with no prior fermentation exposure (as described in current the chapter) allowed for rapid assessment of multiple temperatures and its effects upon yeast behaviour. However, recycling of spent yeast biomass in the brewery encompasses storage of yeast slurries following a fermentation cycle. Thus, for further experiments W34/70 was cropped following an initial fermentation and the impact of storage temperature was investigated (Chapter 5).

# CHAPTER 5: IMPACT OF TEMPERATURE ON GLOBAL YEAST TRANSCRIPTOME DURING CROPPED SLURRY STORAGE

## 5.1 Introduction

The elucidation of the genome sequence of the model laboratory yeast, *Saccharomyces cerevisiae* (Mewes *et al.*, 1997) was key for permitting comprehensive genome-level characterizations of yeast responses to a number of different environmental conditions. Not only did this provide an insight into the complexity of the genome by facilitating the functional assignment of many genes but it also formed the basis of the many elegant tools now at the yeast cell biologist's disposal. Arguably, the most commonly used amongst these involves transcriptional analysis, routinely achieved through the use of microarrays. Although microarrays allow monitoring of the expression of individual genes, it is their capacity to simultaneously assess the expression of thousands of genes which is critical.

### 5.1.1 Oligonucleotide microarrays

Oligonucleotide (oligo) arrays are a class of microarrays which are widely used to evaluate transcript abundance and concomitant gene expression profiles (Chaturvedi *et al.*, 1997, Albertyn *et al.*, 1994a). Oligo arrays designed for evaluating gene expression utilize only the information of gene sequences without any other physical intermediates such as PCR products, clones or similar aids. Their functioning is primarily based on the targeted design of sets of probes to specifically monitor expression of individual genes (Luyten *et al.*, 1994). Each probe is a unique 25-mer oligonucleotide and is independent of other probes within one probe set (Albertyn *et al.*, 1994a, Luyten *et al.*, 1994). The combined information generated from the hybridization efficiency of

different probes in a probe set allows accurate determination of transcript abundance within a sample. GeneChip technology is a type of commercially available and extensively validated oligo arrays. Apart from using probe sets, GeneChip introduces an additional level of stringency by the use of 11 to 20 different probe-pairs in a single probe set. Each probe-pair consists of a perfect-match (PM) and a mismatch (MM) probe (Albertyn *et al.*, 1994a, Luyten *et al.*, 1994). Whilst the PM probe is fully complementary to the target transcript, its corresponding MM has a single mismatch at the 13$^{th}$ base (the rest of the sequence being identical to PM) (Albertyn *et al.*, 1994a, Luyten *et al.*, 1994). Transcript levels can be calculated by simply using the PM data or by extrapolating the hybridization differences between the PM and MM probes across a probe set. Thus, MM probes act as specificity controls and function in distinguishing true-hybridization events from non-specific or semi-specific hybridizations.

Currently, the use of Affymetrix oligonucleotide arrays (also known as GeneChip arrays) are mostly confined to haploid laboratory model organisms such as the *S.cerevisiae* strains S288C and BY4741. Lager yeast strains belong to the species *Saccharomyces pastorianus* which is a hybrid species derived from *S.cerevisiae* and *S.bayanus* and contains two nuclear sub-genomes originating from both parent species (Nakao *et al.*, 2009, Smart, 2007). Although a high degree of sequence homology (up to 100% identity) exists between the *S.cerevisiae* derived sub-genome and the published S288C genome sequence, sequence divergence between the *S.bayanus* derived sub-genome and S288C sequence is considerably higher (75 – 85 % identity)(Casaregola *et al.*, 2001, Pahlman *et al.*, 2001). Moreover, a small

number of lager yeast specific genes are also present in *S.pastorianus* which are absent in the known genome sequences of *S.cerevisiae* and *S.bayanus* (Nakao *et al.*, 2009) and that may result from mosaic genes (Smart, 2007). As a result, direct application of oligonucleotide arrays designed using *Saccharomyces cerevisiae* genome sequence might cause inefficient hybridization of lager brewing yeast transcripts to oligonucleotide probes on account of sequence polymorphisms. This can potentially attenuate the overall signal generated across a probe set and thus reduce the specificity and consequently the quality of information obtained from GeneChip arrays when applied to heterologous species. In order to circumvent the above problem, the method of Xspecies or cross-species hybridization was adopted (Albertyn *et al.*, 1994a, Chaturvedi *et al.*, 1997, Pavlik *et al.*, 1993). Such a bioinformatics approach has improved the sensitivity of *S.cerevisiae* high-density oligonucleotide arrays for the transcriptomic analysis of lager brewing yeast *S.pastorianus W34/70* (Gibson *et al.*, 2010, Gibson *et al.*, 2008).

## 5.2 Yeast Cold Stress Response

Brewing yeast is exposed to a number of environmental changes during industrial applications. Whilst some of the adverse environment driven changes might be transient, other long term environmental changes might involve transcriptional changes whose manifestation is crucial for cellular adaptation (Schade *et al.*, 2004). As mentioned earlier (Section 1.6, Chapter 1), *S. cerevisiae* demonstrates considerable changes in gene expression following a drop in ambient temperature (Lashkari et al, 1997; Sahara et al, 2002, Schade et al, 2004; Murata et al, 2006). The transcriptional response of yeast during batch, fed-batch and continuous fermentations are well documented (Bell *et*

*al.*, 1992, Zaragoza *et al.*, 2002, Van Dijck *et al.*, 2002, Zaragoza *et al.*, 2001, Devirgilio *et al.*, 1993). In addition transcriptional analysis during brewing fermentations at laboratory (Dombek *et al.*, 1999, Posas *et al.*, 1993, Bell *et al.*, 1998) and industrial (Gibson *et al.*, 2008, Gibson *et al.*, 2007, Zhang *et al.*, 1995) scale have been reported. However transcriptional analysis of lager brewing yeast during anaerobic cold storage has not yet been reported. In this chapter this omission has been addressed at laboratory scale for the lager strain W34/70.

## 5.3 RESULTS

In order to assess the impact of storage temperature on G1 (yeast that has completed one fermentation) harvested (cropped) yeast, a freshly propagated culture was pitched into wort to conduct its first (G0) fermentation (G0F) (Section 2.10.2, Chapter 2). Following the completion of G0F, yeast was cropped and the resultant slurry was stored at two different temperatures, 10 and 4 °C (Section 2.10.3, Chapter 2).

### 5.3.1 G0F Profile

During the G0F samples were recovered at 0, 14, 24, 30, 40, 50, 74, 89, 96, 98, 120, 144, 148, 212 and 227 hrs following pitching. Samples were analysed for various cellular and fermentation characteristics such as cell viability (Section 2.11.1, Chapter 2), budding index (Section 2.11.3, Chapter 2), cell density (Section 2.11.4, Chapter 2), sugar utilization (Section 2.18, Chapter 2), ethanol formation and wort attenuation (Section 2.17, Chapter 2) to monitor the progression of fermentation.

Temperature, pH and dissolved oxygen (DO) profiles during G0F are shown in Figure 5-1. The majority of the oxygen was utilized during the first 2 hrs of fermentation, with no oxygen detected at 4 hrs post pitching. A gradual decline in pH was observed until 110 hrs after which this parameter was stable. The temperature within the three G0F vessels matched the specification required for the industrial fermentation that was being mimicked.

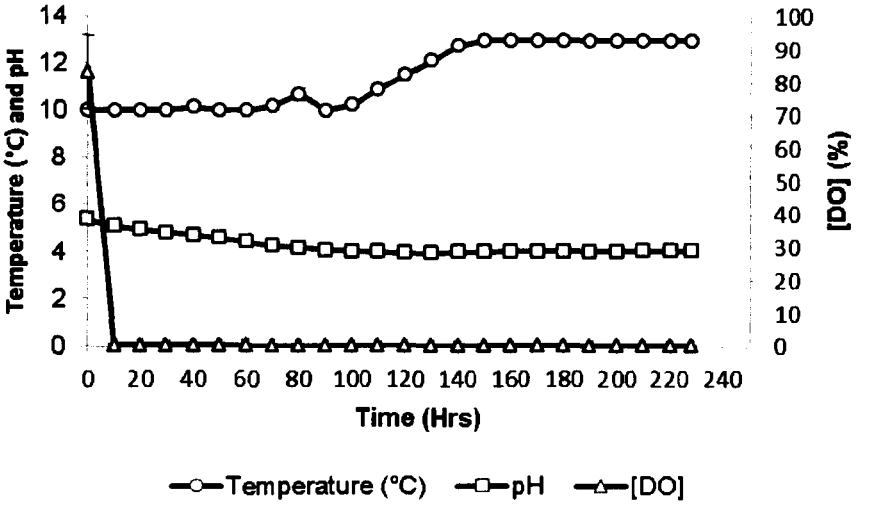



**Figure 5.1 - Fermentation progression as indicated by temperature, pH and dissolved oxygen concentrations ([DO]). Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

### 5.3.1.1 Cellular and fermenting wort key performance indicators during fermentation

The fermentation lag phase with respect to the cell density profile lasted for approximately 14 hrs after which a steady increase in cell density was observed with the onset of stationary phase occurring after 98 hrs of G0F (Figure 5.2). Cell viability was consistently above 96% (Figure 5.2) throughout the duration of G0F. Peak budding index (78%) was observed after the first 30 hrs fermentation and then declined until the proportion of cells reached a minor fraction (7%) of the whole population.

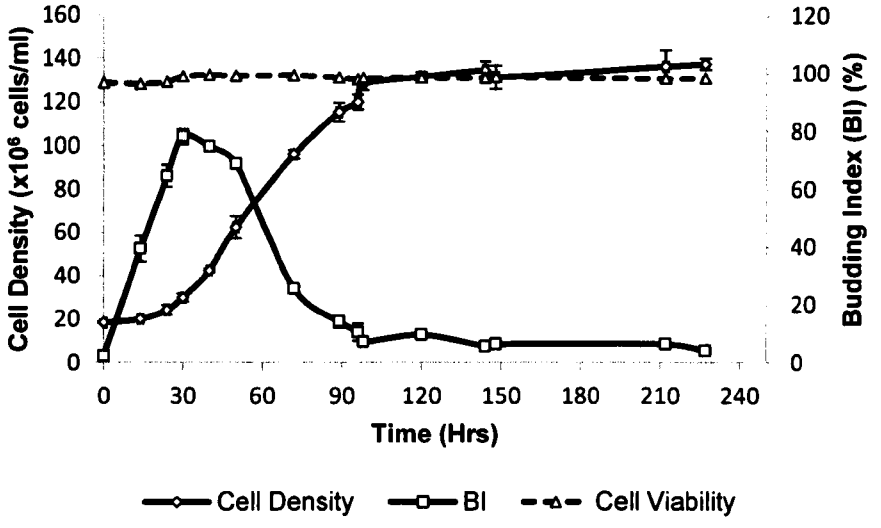



**Figure 5.2 - Yeast cell viability, density and budding index (BI) during G0F. Cell viability was measured using citrate methylene violet (CMV). Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

Specific gravity declined most rapidly during the logarithmic phase of yeast growth (between 30 to 98 hrs) and was an accompanied by an accumulation of ethanol (Figure 5.3) which peaked at 144 hrs.

Maltose, maltotriose, glucose and fructose were the major fermentable sugars with initial wort concentrations of 93 ± 1.5 g / L, 19 ± 1.0 g / L, 17 ± 0.4 g / L, 3 ± 0.1 g / L respectively. Glucose was the first sugar to be utilized and levels dropped during the first 50hrs of G0F (Figure 5.4). Fructose was removed from the wort simultaneously and both monosaccharides were undetectable after 50 - 74 hrs G0F. Exhaustion of monosaccharides triggered the utilization of the relatively abundant di- and tri-saccharide maltose and maltotriose respectively (Figure 5.4). Both the sugars were utilized simultaneously, although maltose consumption was at a comparatively higher rate.

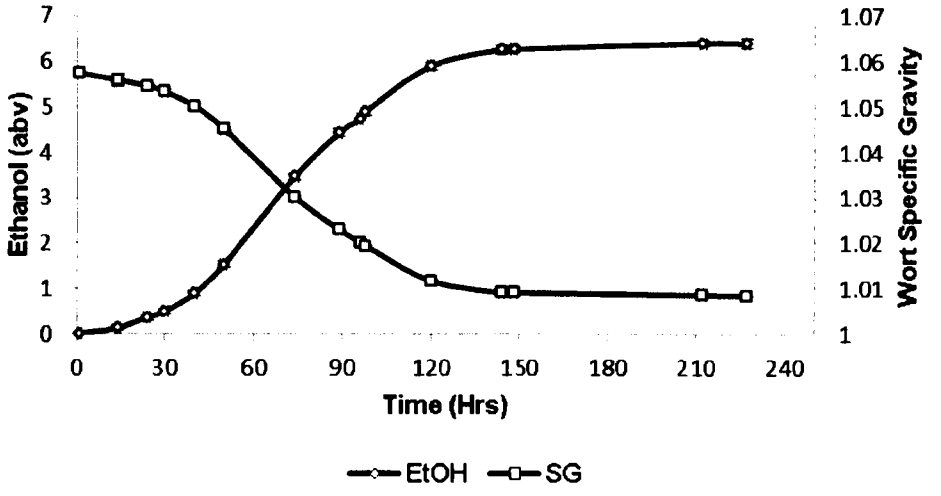



**Figure 5.3 - Specific gravity and ethanol levels during G0F. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

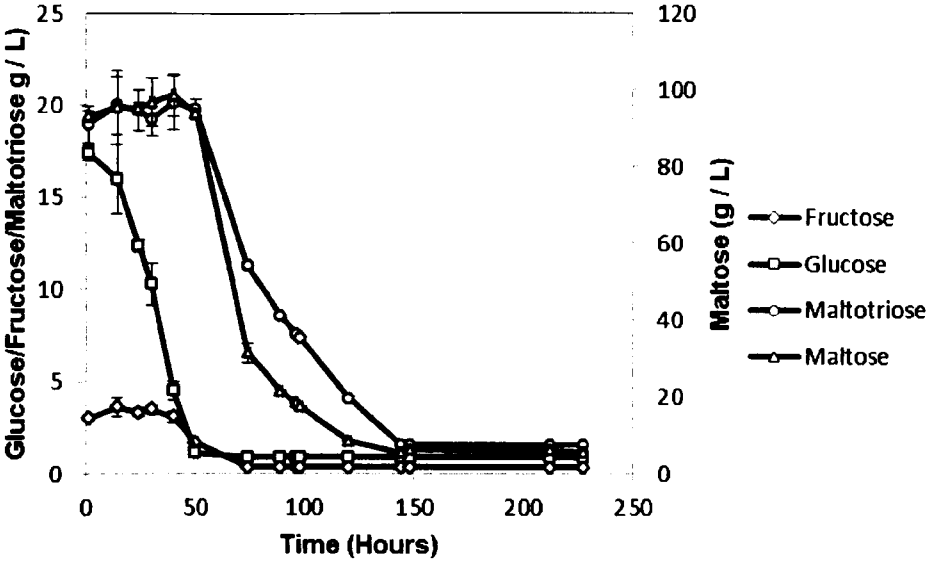



**Figure 5.4 - Utilization of the major fermentable wort sugars during G0F. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

### 5.3.2 Impact of temperature on global yeast transcription during cropped slurry storage

#### 5.3.2.1 Cross-species hybridization

Xspecies or cross species hybridisation has been previously utilized to increase sensitivity and specificity (Chaturvedi *et al.*, 1997, Albertyn *et al.*, 1994a). It involves hybridization of genomic DNA (gDNA) from a target species; PM probes from *S.cerevisiae* were hybridized to *S.pastorianus* gDNA and generate varied amounts of intensities depending on the extent of sequence polymorphism observed. The threshold of gDNA hybridization intensity was progressively increased using 18 probe mask files generated using a parser script (written in Pearl programme) as shown in Figure 5.5. As a result, PM probes below the set user-defined arbitrary threshold were discarded thereby decreasing the number of retained PM probes. However, since a minimum of only one PM probe is required to retain a probe-set (representing a particular transcript), the retention rate for probe sets was less sensitive to increase in gDNA hybridization threshold. The probe mask file with a gDNA intensity threshold of 40 and 60 did not affect estimates of transcript abundance (Figure 5.5); only 7 and 537 out of 10762 available probe sets were excluded from the two probe mask files respectively amounting to less than 5% of the genome coverage. Probe mask files with intensity thresholds of 80 and beyond lead to exclusion of 15% and more of the overall *S.pastorianus* genome (Figure 5.5). Thus probe pairs with a gDNA hybridization intensity of 60 and covering around 95% of the genome were selected for subsequent transcriptome analysis of cold stored *S.pastorianus* W34/70 yeast slurry.

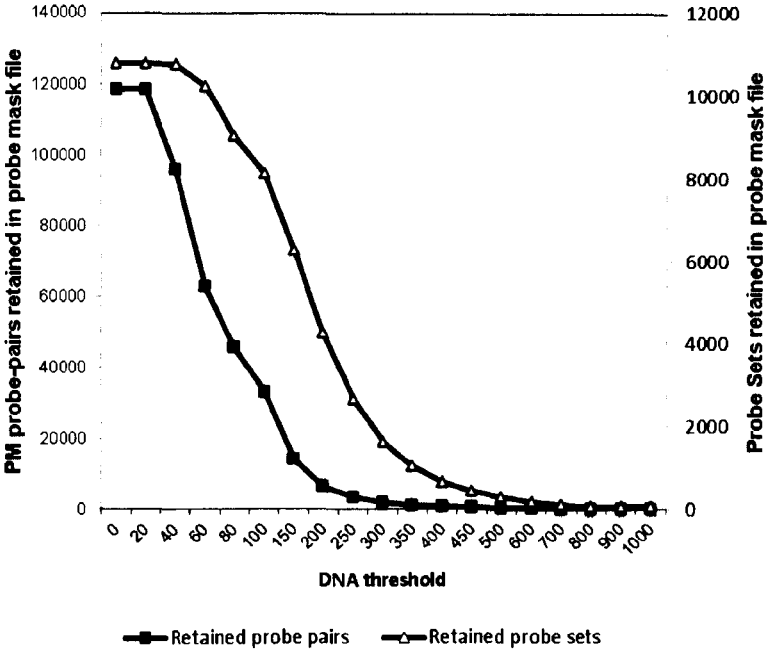
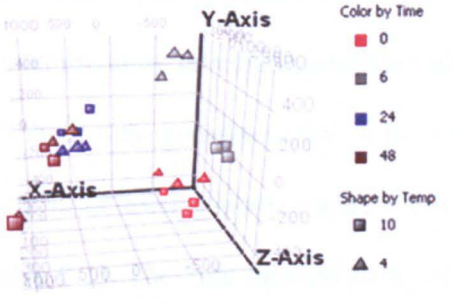



**Figure 5.5 - Number of *S. cerevisiae* probe-pairs and probe-sets from the GeneChip®Yeast Genome 2.0 Array as a function of the gDNA hybridisation intensity thresholds used to generate the probe mask files. The retention rate for the probe-pairs and the probe-sets used in the probe mask files has been indicated in the legend. Data were obtained by hybridising genomic DNA from *S. Pastorianus* to the the GeneChip®Yeast Genome 2.0 Array.**

### 5.3.2.2 Quality Control Analysis of Oligonucleotide Arrays

The principal component analysis (PCA) scores for replicate samples stored at 10 and 4 °C is shown in Figure 5.6a in a 3-D scatter plot. Such a plot is ideal for viewing separation between groups of replicates. Triplicate samples obtained after 0, 6 and 24 hr of storage at 10 and 4°C appeared to form distinct groups on the PCA plot. Relatively higher divergence in PCA scores seemed to exist for the 48 hr replicates for both conditions. As a result, Pearson correlation coefficient values were also considered (Figure 5.6b). All of the replicates had a correlation coefficient of 0.98 or more indicating high experimental reproducibility and were deemed acceptable for further analysis.

(a)




(b)

| Storage Sample Replicate | Correlation Coefficient | | |
|---|---|---|---|
| 0h_10C_Rep1_Yeast.CEL | 1.00 | 0.99 | 1.00 |
| 0h_10C_Rep2_Yeast.CEL | 0.99 | 1.00 | 0.99 |
| 0h_10C_Rep3_Yeast.CEL | 1.00 | 0.99 | 1.00 |
| 0h_4C_Rep1_Yeast.CEL | 1.00 | 0.99 | 0.99 |
| 0h_4C_Rep2_Yeast.CEL | 0.99 | 1.00 | 1.00 |
| 0h_4C_Rep3_Yeast.CEL | 0.99 | 1.00 | 1.00 |
| 6h_10C_Rep1_Yeast.CEL | 1.00 | 0.99 | 1.00 |
| 6h_10C_Rep2_Yeast.CEL | 0.99 | 1.00 | 0.99 |
| 6h_10C_Rep3_Yeast.CEL | 1.00 | 0.99 | 1.00 |
| 6h_4C_Rep1_Yeast.CEL | 1.00 | 0.99 | 0.99 |
| 6h_4C_Rep2_Yeast.CEL | 0.99 | 1.00 | 0.98 |
| 6h_4C_Rep3_Yeast.CEL | 0.99 | 0.98 | 1.00 |
| 24h_10C_Rep1_Yeast.CEL | 1.00 | 0.99 | 1.00 |
| 24h_10C_Rep2_Yeast.CEL | 0.99 | 1.00 | 1.00 |
| 24h_10C_Rep3_Yeast.CEL | 1.00 | 1.00 | 1.00 |
| 24h_4C_Rep1_Yeast.CEL | 1.00 | 0.99 | 1.00 |
| 24h_4C_Rep2_Yeast.CEL | 0.99 | 1.00 | 0.99 |
| 24h_4C_Rep3_Yeast.CEL | 1.00 | 0.99 | 1.00 |
| 48h_10C_Rep1_Yeast.CEL | 1.00 | 0.99 | 0.99 |
| 48h_10C_Rep2_Yeast.CEL | 0.99 | 1.00 | 0.99 |
| 48h_10C_Rep3_Yeast.CEL | 0.99 | 0.99 | 1.00 |
| 48h_4C_Rep1_Yeast.CEL | 1.00 | 0.99 | 0.98 |
| 48h_4C_Rep2_Yeast.CEL | 0.99 | 1.00 | 0.99 |
| 48h_4C_Rep3_Yeast.CEL | 0.98 | 0.99 | 1.00 |

**Figure 5.6 – (a) Plot of Principal Component Analysis (PCA) using the microarray expression data. PCA scores have been plotted against three components designated as x-axis (55.72%), y-axis (20.51%) and z-axis (14.13%). Each point represents an array whose shape and colour code represents the storage temperature and sampling time-point. (b) Table depicting the Pearson correlation coefficients for the various microarray replicates for each of the sampling-time points.**

#### 5.3.2.3 Variation in global transcriptome during cropped slurry storage at 4 °C

An overview of the differential gene expression data during *W34/70* slurry storage at 4°C is shown in Table 5-1. Differential expression was deemed to have occurred when the transcript levels changed by>2 fold at a significance of $p < 0.05$ as assessed by One way ANOVA with Benjamini-Hochberg (BH) false discovery rate (FDR) correction. Following Xspecies based probe selection using a gDNA hybridization intensity threshold of 60, probe sets of 866 genes (out of 5798 entities) encompassing 14.9% of W34/70 genome demonstrated significant regulation. In comparison at the start of storage (0hrs), 282 entities (4.9% of the total genome) demonstrated significant changes in transcript abundance ($p < 0.05$) when compared to that observed after 6 hrs of cold incubation at 4°C. Further storage of W34/70 slurry under at this temperature revealed significant alterations in the expression of 422 ORFs (7.3 % of total genome) after 24 hrs whilst the transcript levels of 671 entities (11.6 % of total genome) were modulated by 2 fold or higher after 48 hrs (Table 5-1). The bulk (around 66%) of the annotated ORFs was induced in the first 6 hrs of slurry storage coinciding with a down-regulation of the remaining 34% of the genes (Table 5-1). Increased storage duration seemed to reverse this scenario with the majority of the ORFs being repressed after 24 hrs (61%) and 48 hrs (60%) of storage (Table 5-1).

**Table 5-1 - Number of ORFs demonstrating differential transcript abundance over 48 hrs of anaerobic W34/70 slurry maintenance at 4 °C. Gene induction or repression is based on comparison with yeast sampled at 0 hr. Significance for differential expression was defined as $p < 0.05$ (One Way ANOVA with Benjamini Hochberg correction) and a fold change of >2.**

| | Storage Time | | | | | |
|---|---|---|---|---|---|---|
| | 6h | (%) | 24h | (%) | 48h | (%) |
| Total Regulated ORFS | 282 | 4.9 | 422 | 7.3 | 671 | 11.6 |
| Induced ORFs | 187 | 66 | 166 | 39 | 271 | 40 |
| Repressed ORFs | 95 | 34 | 256 | 61 | 400 | 60 |

Almost 58% (163 out of 282) of the total genes that were differentially expressed after 6 hrs of cropped yeast storage did not exhibit modified transcript abundance following extended storage (Figure 5.7a). Analysis of transcript variation between successive sampling points (0, 6 24 and 48 hrs) also indicated that the bulk of the changes in gene expression (78%; 220 out of a total 282) occurring during the first 6 hours at 4 °C was confined to the initial storage stages (Figure 5.7b). This suggests that the majority of the initial transcriptome modulation is specifically aimed at immediate cellular adaptation following transfer of yeast populations from fermentation conditions to the low temperature storage environment. Prolonged storage seemed to necessitate additional adaptation with 7.9 % (458 out of 5798) of W34/70 ORFS being differentially expressed between 6 and 24 hrs (Figure 5.7b). After 24 hours further cold incubation resulted in little difference in yeast transcriptional status since only 0.1% (4 of total) of the genome was differentially expressed (Figure 5.7b).

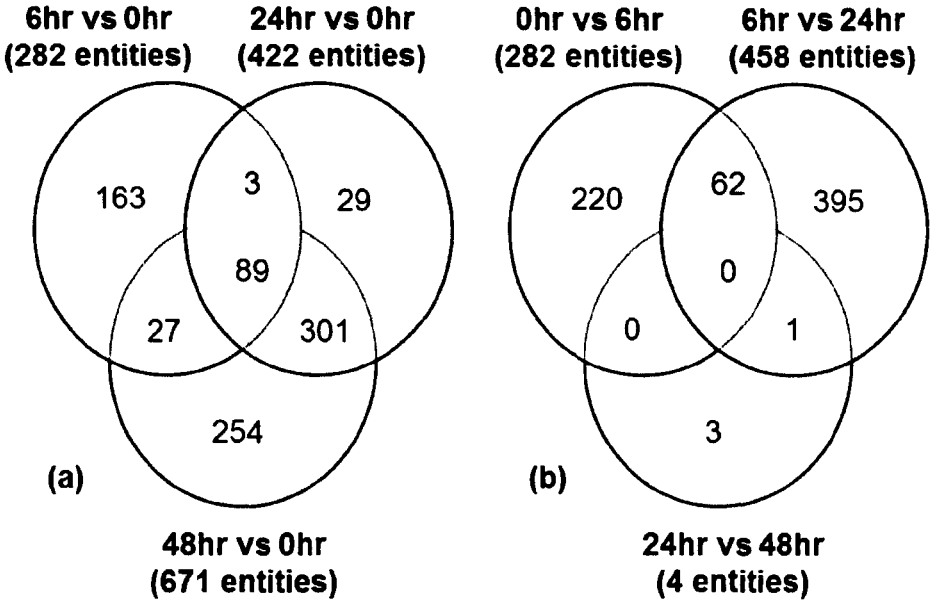



**Figure 5.7 – Global transcriptome response during anaerobic storage at 4 °C. (a) Venn diagrams show a comparison of the significant differentially expressed genes (a) between the 6, 24 and 48 hr sampling points and 0hr sample and (b) between successive sampling points.**

#### 5.3.2.3.1 K Means Cluster Analysis of differentially expressed genes

Previously isolated 866 ORFs which were differentially expressed (>2 fold change; $p < 0.05$ after BH-FDR correction) over 48 hrs of W34/70 slurry storage at 4 °C were separated into different groups using K Means cluster analysis. K Means clustering requires manual assignment of the grouping number for assortment of the gene list. Whilst higher number of clusters favours extraction of specific expression patterns, lower cluster number results in larger gene sets per cluster and helps in extraction of specific annotations for gene functional categories (Bell *et al.*, 1992). Thus four clusters were appropriately established using GeneSpring GX, Version 11 (see Figure 5.8).

Cluster 1 contained 80 genes and constituted almost 9.2% of the differentially expressed ORFs during traditional slurry storage. The genes in

this cluster were down-regulated in the first 6 hrs of storage followed by marginal induction to median expression between 6 and 24 hrs (Figure 5.8a). Cluster 2 contained 140 genes (16.0 % of total) which attained peak expression after 6 hrs of slurry storage, followed by repression (Figure 5.8b). Cluster 3 contained the maximum number of genes (385) amongst the 4 groups and constituted almost 45% of the differentially expressed ORFs. Largely, the genes in this cluster were chronically repressed during the entire course of storage with lowest expression levels after 48hrs (Figure 5.8c). The final ORF cluster (cluster 4) comprised 261 genes (29.9% of total) and typically demonstrated a gradual increase in transcript abundance until 24hrs of slurry storage had been completed (Figure 5.8d).

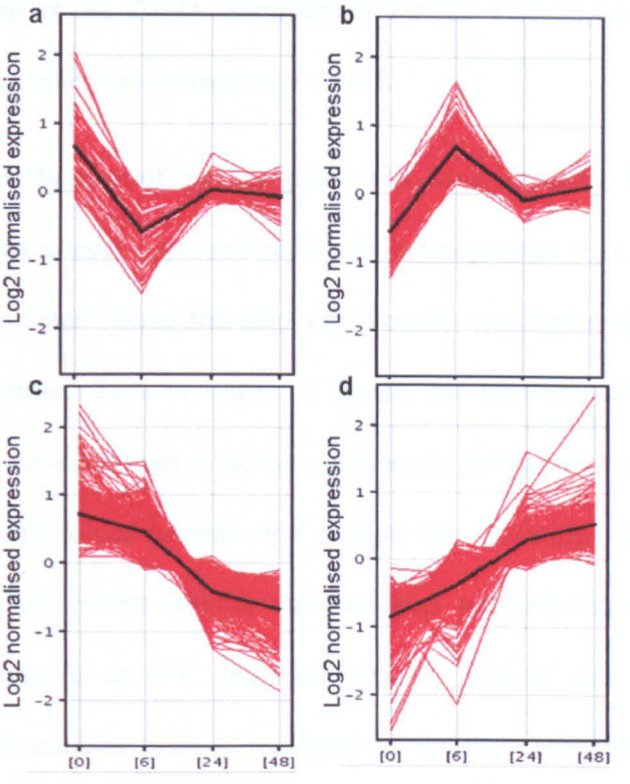



**Figure 5.8 – K-means cluster analysis of genes displaying significant differential expression during cropped slurry storage at 4 °C. A total of 866 genes of known function were divided into 4 clusters: (a) Cluster 1 containing 80 genes, (b) Cluster 2 containing 140 genes, (c) Cluster 3 containing 385 genes and (d) Cluster 4 containing 261 genes. The expression intensity refers to the $log_2$ values of the normalized data.**

#### 5.3.2.3.2 MIPS-FunCat Classification of K Means clusters

Functional annotation of the four K-Means clusters was performed using MIPS-Functional Catalogue (FunCat), a functional annotation scheme developed at the Munich Information Centre for Protein Sequences (MIPS) (Ruepp *et al.*, 2004). MIPS - FunCat has a hierarchically structured and controlled vocabulary which classifies transcriptomic data into 28 major categories and several downstream sub-categories to form a "tree-like"

structure (Ruepp *et al.*, 2004). Significance for the various over- or under-represented categories was defined as $p < 0.05$.

MIPS classification of various genes in K-Means Cluster 1 is shown in (Table 5-2). This cluster appeared relatively heterogenous with "cell rescue, defence and virulence" being the only significantly enriched major functional category. It formed 23% of cluster 1 genes, the majority of which were associated with cellular stress responses such as oxidative stress, accumulation of unfolded or mis-folded proteins and cellular detoxification via the catalase reaction. Other prominent cellular sub-functions transcriptionally repressed during early storage at 4°C included protein folding and stabilization, metabolism and trans-membrane transport of reserve carbohydrates/organic compounds and signal transduction via serine/threonine kinase receptor activation (Table 5-2). Cluster 1 also had a high number of ORFs (almost 27%) with no documented function (data not shown).

**Table 5-2 – Functional classification of 80 differentially transcribed genes within K-Means Cluster 1 using the MIPS Functional Catalogue (Ruepp *et al.*, 2004). The cut off for the enrichment magnitude was $p < 0.05$.**

| MIPS FUNCTIONAL CATEGORY - Cluster 1 | Group Occurence (%) | Genome occurence (%) | P-VALUE |
|---|---|---|---|
| **02 ENERGY** | | | |
| 02.01 glycolysis and gluconeogenesis | | | |
| 02.01.01 glycolysis methylglyoxal bypass | 1 | 0.0 | 1.2E-02 |
| 02.19 metabolism of energy reserves (e.g. glycogen, trehalose) | 4 | 0.9 | 3.1E-02 |
| 14.01 protein folding and stabilization | 8 | 1.5 | 9.0E-04 |
| **20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES** | | | |
| 20.01 transported compounds (substrates) | | | |
| 20.01.03 C-compound and carbohydrate transport | 4 | 1.0 | 4.1E-02 |
| **30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM** | | | |
| 30.05 transmembrane signal transduction | | | |
| 30.05.01.18 serine/threonine kinase signalling pathways | 1 | 0.0 | 2.4E-02 |
| **32 CELL RESCUE, DEFENSE AND VIRULENCE** | 23 | **9.0** | **2.9E-04** |
| 32.01 stress response | 21 | 7.3 | 8.1E-05 |
| 32.01.01 oxidative stress response | 4 | 0.9 | 2.9E-02 |
| 32.01.07 unfolded protein response (e.g. ER quality control) | 5 | 1.1 | 9.8E-03 |
| 32.07.07.01 catalase reaction | 1 | 0.0 | 2.4E-02 |
| **42 BIOGENESIS OF CELLULAR COMPONENTS** | | | |
| 42.01 cell wall | 8 | 3.5 | 4.6E-02 |

Group occurrence - number of genes in the cluster belonging to the specified functional category; Genome occurrence - number of genes in the genome belonging to the specified functional category.

Protein synthesis and transcription were the most over-represented cellular functions in K-Means Cluster 2, featuring 39% and 35% of the genes (Table 5-3). Protein synthesis was primarily directed towards ribosome biogenesis with induction of both ribosomal protein large subunit (*RPL14B*, *RPL21B*, *RPL22A*, *RPL25*, *RPL27A*, *RPL31B*, *RPL33B*, *RPL37A*, *RPL39*, *RPL8A*, *RPL9B*) and small subunit (*RPA12*, *RPA34*, *RPA43*, *RPA49*) genes (Fatica and Tollervey, 2002, Lempiainen and Shore, 2009, Kressler *et al.*, 2010). Within transcription-related processes, the synthesis, processing and modification of ribosomal RNA (rRNA) were significantly enriched (Table 5-3). rRNA processing encompassed genes encoding factors for 35S rRNA maturation (*RPP1*, *RRP3*), synthesis of 90S pre-ribosome (*CBF5* and *DIM1*) and small subunit processosome (SSU) components (*BUD21*, *IMP3*, *UTP21*), RNA helicases (*DBP7*), ribosomal subunit export from nucleus proteins

(*RIX1*), large ribosomal subunit L7 protein (*RLP7*) and 60S ribosome subunit biogenesis (*NIP7*) (Fatica and Tollervey, 2002, Lempiainen and Shore, 2009, Kressler *et al.*, 2010). rRNA synthesis was predominantly driven by RNA polymerase I, due to induction of RNA polymerase subunit encoding genes *RPA34*, *RPA12*, *RPA49*, *RPA43* (Fatica and Tollervey, 2002, Lempiainen and Shore, 2009, Kressler *et al.*, 2010). Genes involved in nucleotide/nucleobase (*AAH1*, *URA7*, *URA6*, *IMD4*) and pseudouridine (*CBF5* and *PUS1*) synthesis were also significantly over-represented in cluster 2 (Table 5-3). Pseudouridnines are required for site-specific maturation of rRNA through covalent interactions. Thus, both of these above probably act as raw materials for RNA synthesis and complements ribosome biogenesis.

**Table 5-3 – Functional classification of 140 differentially expressed genes within K-Means cluster 2 using the MIPS Functional Catalogue (Ruepp *et al.*, 2004). Cut off for the enrichment magnitude was $p < 0.05$.**

| MIPS FUNCTIONAL CATEGORY - Cluster 2 | Group Occurence (%) | Genome occurence (%) | P-VALUE |
|---|---|---|---|
| **01 METABOLISM** | | | |
| 01.03 nucleotide/nucleoside/nucleobase metabolism | 8 | 3.7 | 1.1E-02 |
| 01.03.04 pyrimidine nucleotide/nucleoside/nucleobase metabolism | 4 | 0.8 | 6.1E-04 |
| **11 TRANSCRIPTION** | 35 | 17.5 | 4.2E-07 |
| 11.02.01 rRNA synthesis | 6 | 1.4 | 4.6E-04 |
| 11.02.02 tRNA synthesis | 4 | 0.6 | 1.4E-03 |
| 11.04 RNA processing | 24 | 7.1 | 1.8E-10 |
| 11.04.01 rRNA processing | 22 | 3.3 | 3.7E-17 |
| 11.06 RNA modification | 8 | 1.0 | 1.2E-07 |
| 11.06.01 rRNA modification | 4 | 0.3 | 1.6E-06 |
| 11.06.02 tRNA modification | 3 | 0.7 | 1.5E-02 |
| **12 PROTEIN SYNTHESIS** | 39 | 7.8 | 8.3E-25 |
| 12.01 ribosome biogenesis | 35 | 5.1 | 6.5E-28 |
| 12.01.01 ribosomal proteins | 26 | 4.0 | 1.4E-20 |
| 12.04.01 translation initiation | 3 | 0.7 | 1.1E-02 |
| **16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT (structural** | 29 | 17.1 | 2.3E-04 |
| 16.03 nucleic acid binding | 22 | 5.6 | 3.6E-11 |
| 16.03.01 DNA binding | 7 | 2.6 | 8.6E-03 |
| 16.03.03 RNA binding | 15 | 3.1 | 7.6E-10 |

Group occurrence - number of genes in the cluster belonging to the specified functional category; Genome occurrence - number of genes in the genome belonging to the specified functional category.

Cluster 3 was populated with genes involved in a myriad of cellular processes (Table 5-4 & Table 5-5). Cell metabolism constituted the largest fraction (30% of cluster 3 ORFs) (see Table 5-4) and various genes associated with metabolic reactions of amino acids (especially urea, cysteine and tryptophan), nitrogenous compounds, pyrimidines (particularly thiamine biosynthesis encoded by *THI5, THI12, THI13*) and cellular carbohydrates (particularly carbohydrate synthesis) were down-regulated (Table 5-4). ATP biosynthesis was another significantly represented cellular process encompassing 7 genes which encode different subunits of mitochondrial ATP synthase (*ATP4*, *ATP7*, *ATP14*, *ATP5*, *ATP19*, *ATP3*, *ATP16*) (Table 5-4). Abundance of genes associated with electron transport/membrane-associated energy conservation and alcohol fermentation (denoting ethanol biosynthesis) was also observed (Table 5-4).

**Table 5-4 – Functional classification of 385 differentially expressed genes within K-Means cluster 3 using the MIPS Functional Catalogue (Ruepp *et al.*, 2004). The cut off for the enrichment magnitude was $p < 0.05$ (continued later).**

| MIPS FUNCTIONAL CATEGORY - Cluster 3a | Group Occurence (%) | Genome occurence (%) | P-VALUE |
|---|---|---|---|
| **01 METABOLISM** | 30 | 24.6 | 8.3E-03 |
| 01.01 amino acid metabolism | 7 | 4.0 | 6.2E-03 |
| 01.01.05.03 metabolism of urea (urea cycle) | 1 | 0.1 | 4.7E-02 |
| 01.01.09 metabolism of the cysteine - aromatic group | 3 | 1.3 | 8.4E-03 |
| 01.01.09.03 metabolism of cysteine | 1 | 0.2 | 4.0E-02 |
| 01.01.09.06 metabolism of tryptophan | 1 | 0.3 | 3.0E-02 |
| 01.02 nitrogen, sulfur and selenium metabolism | 5 | 1.6 | 1.6E-05 |
| 01.02.02 nitrogen metabolism | 1 | 0.1 | 1.5E-05 |
| 01.02.02.09 catabolism of nitrogenous compounds | 1 | 0.1 | 1.5E-05 |
| 01.03.04 pyrimidine nucleotide/nucleoside/nucleobase metabolism | 2 | 0.8 | 2.4E-02 |
| 01.05 C-compound and carbohydrate metabolism | 11 | 8.2 | 3.1E-02 |
| 01.05.03 polysaccharide metabolism | 2 | 1.1 | 4.2E-02 |
| 01.05.06 C-2 compound and organic acid metabolism | 1 | 0.1 | 1.4E-02 |
| 01.05.06.07 C-2 compound and organic acid catabolism | 1 | 0.1 | 1.4E-02 |
| 01.20.17 metabolism of secondary products derived from primary amino acids | 1 | 0.3 | 2.5E-02 |
| **02 ENERGY** | 9 | 6.0 | 1.4E-02 |
| 02.11 electron transport and membrane-associated energy conservation | 2 | 1.0 | 3.0E-02 |
| 02.16 fermentation | 2 | 0.8 | 2.2E-02 |
| 02.16.01 alcohol fermentation | 1 | 0.2 | 4.0E-02 |
| 02.45 energy conversion and regeneration | 3 | 0.7 | 2.3E-04 |
| 02.45.15 energy generation (e.g. ATP synthase) | 2 | 0.3 | 1.6E-04 |
| **10 CELL CYCLE AND DNA PROCESSING** | | | |
| 10.01 DNA processing | | | |
| 10.01.03.05 extension/ polymerization activity | 2 | 0.6 | 2.3E-02 |
| 10.03 cell cycle | | | |
| 10.03.01.01.11 M phase | 2 | 0.8 | 1.1E-02 |
| 10.03.04 nuclear and chromosomal cycle | 3 | 1.8 | 1.6E-02 |
| 10.03.04.09 nuclear migration | 1 | 0.2 | 1.1E-02 |
| **12 PROTEIN SYNTHESIS** | | | |
| 12.10 aminoacyl-tRNA-synthetases | 2 | 0.6 | 8.1E-03 |
| **14 PROTEIN FATE (folding, modification, destination)** | | | |
| 14.10 assembly of protein complexes | 6 | 3.2 | 8.9E-03 |

Group occurrence - number of genes in the cluster belonging to the specified functional category; Genome occurrence - number of genes in the genome belonging to the specified functional category.

Cellular transport was the second most abundantly represented functional category in cluster 3 (23% of genes) (Table 5-5). Amongst the specific substrates whose transport seemed to be repressed with progression of storage at 4 °C included ions (both cations and anions), amino acids, amines/polyamines and peptides (Table 5-5). Mitochondria related transport was the most significantly represented functional sub-category besides vesicle-mediated transport routes (exocytosis and endocytosis) (Table 5-5). Cluster 3 also contained genes which significantly represented certain select cellular

functions. These included DNA synthesis and replication (such as DNA extension/polymerization), cell division related activities (such as M phase of mitotic cell cycle and directed nuclear migration), protein binding and cellular component biogenesis (Table 5-5).

**Table 5-5 - Functional classification of 385 differentially expressed genes within K-Means cluster 3 using the MIPS Functional Catalogue (Ruepp *et al.*, 2004). The cut off for the enrichment magnitude was $p < 0.05$ (continued from before).**

| MIPS FUNCTIONAL CATEGORY - Cluster 3b | Group Occurence (%) | Genome occurence (%) | P-VALUE |
|---|---|---|---|
| **16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT** | 20 | 17.1 | 4.9E-02 |
| 16.01 protein binding | 9 | 6.4 | 1.3E-02 |
| 16.07 structural protein binding | 2 | 0.9 | 1.9E-02 |
| 16.19 nucleotide/nucleoside/nucleobase binding | 6 | 3.7 | 9.2E-03 |
| 16.19.03 ATP binding | 5 | 3.1 | 1.1E-02 |
| **20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND ROUTES** | 23 | 16.9 | 6.1E-04 |
| 20.01 transported compounds (substrates) | 16 | 9.5 | 8.9E-05 |
| 20.01.01 ion transport | 5 | 2.4 | 3.7E-03 |
| 20.01.01.01 cation transport (H+, Na+, K+, Ca2+ , NH4+, etc.) | 4 | 2.0 | 6.7E-03 |
| 20.01.01.07 anion transport | 2 | 0.4 | 6.9E-04 |
| 20.01.07 amino acid/amino acid derivatives transport | 2 | 0.7 | 1.3E-03 |
| 20.01.09 peptide transport | 1 | 0.2 | 2.5E-02 |
| 20.01.11 amine / polyamine transport | 1 | 0.2 | 6.2E-03 |
| 20.03 transport facilities | 5 | 3.2 | 2.8E-02 |
| 20.03.01 channel / pore class transport | 1 | 0.2 | 1.1E-02 |
| 20.03.01.01 ion channels | 1 | 0.2 | 1.9E-02 |
| 20.09 transport routes | 17 | 11.6 | 1.8E-03 |
| 20.09.04 mitochondrial transport | 4 | 1.7 | 4.7E-04 |
| 20.09.16.09 vesicular cellular export | 1 | 0.5 | 4.7E-02 |
| 20.09.16.09.03 exocytosis | 1 | 0.5 | 4.7E-02 |
| 20.09.18 cellular import | 4 | 2.4 | 3.8E-02 |
| **30 CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION** | | | |
| 30.05 transmembrane signal transduction | 2 | 0.7 | 4.4E-02 |
| **32 CELL RESCUE, DEFENSE AND VIRULENCE** | | | |
| 32.01.05 heat shock response | 1 | 0.3 | 3.0E-02 |
| 34 INTERACTION WITH THE ENVIRONMENT | 10 | 7.6 | 2.1E-02 |
| 34.01 homeostasis | 5 | 3.0 | 1.4E-02 |
| 34.01.01 homeostasis of cations | 4 | 2.6 | 3.6E-02 |
| 34.01.01.03 homeostasis of protons | 2 | 0.8 | 7.6E-03 |
| 42 BIOGENESIS OF CELLULAR COMPONENTS | 17 | 14.0 | 4.5E-02 |
| **43 CELL TYPE DIFFERENTIATION** | | | |
| 43.01.03.05 budding, cell polarity and filament formation | 8 | 5.1 | 2.4E-02 |

Group occurrence - number of genes in the cluster belonging to the specified functional category; Genome occurrence - number of genes in the genome belonging to the specified functional category.

MIPS – FunCat annotation of K-Means Cluster 4 revealed cellular transcription to be the most abundant (30%) and significantly represented functional category (Table 5-6). It contained genes known for their

involvement in rRNA processing/modification and transcription repression/termination. This group was also abundant in ORFs encoding proteins with binding function or cofactor requirement (22 % of group occurrence) (Table 5-6). Aspartate and asparagine metabolism along with genes involved in acetate fermentation formed a small fraction in this cluster. Interestingly, nutrient starvation response seemed to be one of the significantly over-represented sub-categories, though it only comprised of three genes (*ASP3-3, ASP3-2* and *ASP3-4*) and constituted a minor fraction (1%) of the group of genes in cluster 4 (Table 5-6).

**Table 5-6 – Functional classification of 261 differentially expressed genes within K-Means cluster 4 using the MIPS Functional Catalogue (Ruepp *et al.*, 2004). The cut off for the enrichment magnitude was $p < 0.05$.**

| MIPS FUNCTIONAL CATEGORY - Cluster 4 | Group Occurence (%) | Genome occurence (%) | P-VALUE |
|---|---|---|---|
| **01 METABOLISM** | | | |
| 01.01.06.01 metabolism of aspartate | 1 | 0.1 | 4.3E-03 |
| 01.01.06.02 metabolism of asparagine | 1 | 0.1 | 4.3E-03 |
| 01.01.06.02.02 degradation of asparagine | 1 | 0.1 | 5.8E-04 |
| 01.03.16 polynucleotide degradation | 3 | 1.4 | 2.0E-02 |
| 01.03.16.01 RNA degradation | 2 | 0.8 | 1.5E-02 |
| 01.07.07 regulation of the metabolism of vitamins, cofactors, and prosthetic groups | 1 | 0.1 | 1.4E-02 |
| **02 ENERGY** | | | |
| 02.16.13 acetate fermentation | 0 | 0.0 | 4.0E-02 |
| **11 TRANSCRIPTION** | **30** | **17.5** | **3.7E-07** |
| 11.02.03 mRNA synthesis | 13 | 9.4 | 4.7E-02 |
| 11.02.03.01.07 transcription termination | 1 | 0.2 | 1.6E-02 |
| 11.02.03.04.03 transcription repression | 2 | 0.5 | 4.4E-03 |
| 11.04 RNA processing | 16 | 7.1 | 9.3E-07 |
| 11.04.01 rRNA processing | 12 | 3.3 | 6.2E-09 |
| 11.06 RNA modification | 4 | 1.0 | 8.5E-04 |
| 11.06.01 rRNA modification | 2 | 0.3 | 5.3E-04 |
| **16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT** | **22** | **17.1** | **3.2E-02** |
| 16.03 nucleic acid binding | 13 | 5.6 | 1.0E-05 |
| 16.03.03 RNA binding | 9 | 3.1 | 5.0E-06 |
| **20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND ROUTES** | | | |
| 20.01.03 C-compound and carbohydrate transport | 2.46 | 1.0 | 3.8E-02 |
| **32 CELL RESCUE, DEFENSE AND VIRULENCE** | | | |
| 32.01.11 nutrient starvation response | 1.23 | 0.2 | 2.0E-02 |

Group occurrence - number of genes in the cluster belonging to the specified functional category; Genome occurrence - number of genes in the genome belonging to the specified functional category.

#### 5.3.2.4 Impact of higher storage temperature on cropped yeast global transcriptome

Following transcriptional assessment of cropped brewing yeast during storage at 4 °C, microarray analysis was further extended to ascertain the impact of an increase in storage temperature. For transcriptome comparison as a function of temperature, fermented slurry samples harvested after 0, 6, 24 and 48 hrs of incubation at 4°C and 10°C were compared (refer to Section 2.16, Chapter 2). Transcript abundance in slurry stored at 10°C was compared with that at 4°C (10°C vs 4°C) at each of the four sampling points treating them as distinct experiments. Criteria for differential expression were defined as a change in gene expression of more than 1.3 fold at significance of $p < 0.05$ (One way ANOVA with BH-FDR correction).

It was earlier demonstrated (Chapter 3) that the cool incubator requires 60 min to cool the yeast slurry's core temperature from 10°C to 4°C. Thus, for slurry storage at 4°C, 0hr sampling point represents yeast harvested after 60 min of cold incubation. None of the ORFs were differentially expressed at the 0 hr sampling point (Table 5-7) suggesting that the time frame was too short to significantly affect cellular transcription at investigative temperatures. 506 genes were found to be differentially regulated after 6 hrs, with increased transcript abundance in 174 entities and reduced transcript numbers in 332 entities (Table 5-7). Only 3 entities were differentially expressed between W34/70 populations after 24 hr whilst no difference in global transcription was evident after 48 hr (Table 5-7).

| Entity comparison 10°C/4°C | Storage Time (Hrs) | | | |
|---|---|---|---|---|
| | 0 | 6 | 24 | 48 |
| Total Regulated ORFs | 0 | 506 | 3 | 0 |
| Induced ORFs | - | 174 | 1 | - |
| Repressed | - | 332 | 2 | - |

**Table 5-7 – Number of genes demonstrating differential expression in cropped W34/70 after the storage temperature was increased from 4 °C to 10 °C. Criteria for differential regulation were $p < 0.05$ (One Way ANOVA with FDR correction) and a >1.3 fold variation in transcript number at 10 °C in comparison to levels at 4 °C.**

Genes demonstrating significantly different regulation after 6hrs of cold storage were annotated using the MIPS FunCat scheme (Ruepp *et al.*, 2004). Amongst the genes with higher transcript abundance, functions associated with cellular energy generation were significantly over-represented (Table 5-8). This was predominantly focused on the activity of the pyruvate dehydrogenase complex for acetyl CoA metabolism, and aerobic respiration. Such an observation together with significant enrichment of certain metabolism-related functions such as vitamin biosynthesis and secondary metabolism (Table 5-8) suggests higher energy requirement for yeast survival in the initial stages of storage at 10 °C. Almost 12% of ORFs with increased transcript abundance after 6hrs of incubation at 10°C were involved in stress responses, particularly cellular detoxification (see Table 5-8). Further MIPS analysis of the genes within the "cell rescue, defence and virulence" category (data not shown) revealed the presence of genes associated with oxidative stress (*GRX1, PST2, FMP46*) and osmotic/salt stress (*MYO3, GCY1, RRD2*). Storage at the higher temperature also seemed to cause relatively higher induction of genes associated with protein modification, auto-proteolysis and reorganisation of the

mitochondrion (Table 5-8). Together these observations might indicate prevalence of increased stress during the initial phases of slurry storage at 10 °C thereby eliciting increased stress responses to cope with the harsher environment.

ORFs which were less abundant when the storage temperature was increased from 4 to 10 °C, were well populated (41.5% of total) with genes associated with cellular transcription, particularly the rRNA machineries (processing, modification and synthesis) (Table 5-8). A large fraction of ORFs (15.9% of total) encoding proteins known to bind to nucleic acids were also present in this group. This suggests reduced cellular efforts to overhaul its transcriptional apparatus when stored at the higher temperature and was seemingly complemented by lower transcript abundance of genes involved in nucleotide/neucleoside/nucleobase metabolism along with nucleo-cytoplasmic transport of proteins and RNA (Table 5-8). Biogenesis of various cellular compartments including cytoskeletal proteins and nuclear membrane were the significantly enriched functions within the ORFs that demonstrated reduced expression following an increase in storage temperature (Table 5-8).

**Table 5-8 - MIPS Functional classification of the differentially transcribed genes after 6 hrs of increasing the cropped yeast storage temperature from 4°C to 10°C. A total 506 genes were divided into groups of induced (174) and repressed (332) genes before functional annotation. Differential expression was defined as a fold change of >1.3 with $p < 0.05$. The cut off for the enrichment magnitude for the functional categories was $p < 0.05$.**

| MIPS Functional Category - 10°C vs 4°C - 6 Hr (continued later) | Up-regulated | | | Down-regulated | | |
|---|---|---|---|---|---|---|
| | Grp Occ | Genome Occ | P - Value | Grp Occ | Genome Occ | P - Value |
| **01 METABOLISM** | | | | | | |
| 01.01.09.07.01 biosynthesis of histidine | | | | 0.91 | 0.24 | 4.24E-02 |
| 01.03 nucleotide/nucleoside/nucleobase metabolism | | | | 6.42 | 3.65 | 7.99E-03 |
| 01.03.01 purin nucleotide/nucleoside/nucleobase metabolism | | | | 2.14 | 0.99 | 4.24E-02 |
| 01.06.06.11 tetracyclic and pentacyclic triterpenes metabolism | | | | 1.52 | 0.58 | 4.06E-02 |
| **01.07 metabolism of vitamins, cofactors, and prosthetic groups** | 7.59 | 2.67 | 1.01E-03 | | | |
| **01.07.01 biosynthesis of vitamins, cofactors, and prosthetic groups** | 6.96 | 1.79 | 1.11E-04 | | | |
| 01.20 secondary metabolism | 3.79 | 1.27 | 1.49E-02 | | | |
| 01.20.27 metabolism of secondary products derived from aspartic acid and threonine | 0.63 | 0.01 | 2.58E-02 | | | |
| **02 ENERGY** | 10.1 | 5.98 | 2.64E-02 | | | |
| **02.08 pyruvate dehydrogenase complex** | 1.89 | 0.08 | 1.62E-04 | | | |
| 02.13.03 aerobic respiration | 3.79 | 1.25 | 1.40E-02 | | | |
| 02.16.09 mixed acid and butanediol fermentation | 0.63 | 0.01 | 2.58E-02 | | | |
| **10 CELL CYCLE AND DNA PROCESSING** | | | | | | |
| 10.01.03.03 ori recognition and priming complex formation | | | | 1.22 | 0.4 | 4.14E-02 |
| 10.03.03 cytokinesis (cell division) /septum formation and hydrolysis | | | | 2.44 | 1.15 | 3.42E-02 |
| **11 TRANSCRIPTION** | | | | 41.5 | 17.5 | 7.29E-26 |
| 11.02.01 rRNA synthesis | | | | 3.97 | 1.35 | 3.97E-04 |
| 11.04 RNA processing | | | | 28.1 | 7.11 | 1.70E-33 |
| 11.04.01 rRNA processing | | | | 23.2 | 3.34 | 2.77E-46 |
| 11.06 RNA modification | | | | 7.03 | 1.04 | 4.92E-14 |
| 11.06.01 rRNA modification | | | | 2.75 | 0.29 | 9.95E-08 |
| 11.06.02 tRNA modification | | | | 3.97 | 0.7 | 1.91E-07 |
| **12 PROTEIN SYNTHESIS** | | | | 18.3 | 7.83 | 1.57E-10 |
| 12.01 ribosome biogenesis | | | | 13.4 | 5.05 | 1.18E-09 |
| 12.10 aminoacyl-tRNA-synthetases | | | | 2.44 | 0.63 | 8.65E-04 |
| **14 PROTEIN FATE (folding, modification, destination)** | | | | | | |
| 14.04 protein targeting, sorting and translocation | 8.22 | 4.56 | 2.80E-02 | | | |
| 14.07.03 modification by phosphorylation, dephosphorylation, autophosphorylation | | | | 4.89 | 3.03 | 3.91E-02 |
| 14.07.11 protein processing (proteolytic) | 4.43 | 1.45 | 7.77E-03 | | | |
| 14.07.11.01 autoproteolytic processing | 1.89 | 0.42 | 2.83E-02 | | | |

| MIPS Functional Category - 10°C vs 4°C - 6 Hr (continued) | (Up-regulated) | | | (Down-regulated) | | |
|---|---|---|---|---|---|---|
| | Grp Occ | Genome Occ | P - Value | Grp Occ | Genome Occ | P - Value |
| **16 PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT** | | | | 32.4 | 17.1 | 2.66E-12 |
| 16.03 nucleic acid binding | | | | 15.9 | 5.56 | 1.72E-12 |
| 16.03.03 RNA binding | | | | 12.5 | 3.09 | 3.51E-15 |
| 16.07 structural protein binding | | | | 2.14 | 0.91 | 2.82E-02 |
| 16.13 C-compound binding | 1.26 | 0.14 | 2.11E-02 | | | |
| 16.13.03 fatty acid binding (e.g. acyl-carrier protein) | 0.63 | 0.01 | 2.58E-02 | | | |
| 16.19 nucleotide/nucleoside/nucleobase binding | | | | 6.72 | 3.67 | 4.07E-03 |
| 16.19.03 ATP binding | | | | 6.11 | 3.11 | 2.77E-03 |
| **20 CELLULAR TRANSPORT, TRANSPORT FACILITIES AND ROUTES** | | | | | | |
| 20.01.10 protein transport | | | | 4.28 | 2.28 | 1.67E-02 |
| 20.01.21 RNA transport | | | | 4.28 | 1.4 | 1.57E-04 |
| 20.09.01 nuclear transport | | | | 5.5 | 1.38 | 3.51E-07 |
| **32 CELL RESCUE, DEFENSE AND VIRULENCE** | 15.8 | 9.03 | 3.77E-03 | | | |
| 32.01 stress response | 12 | 7.34 | 2.18E-02 | | | |
| 32.07 detoxification | 4.43 | 1.9 | 3.09E-02 | | | |
| 32.07.07.03 glutathione conjugation reaction | 1.26 | 0.08 | 6.27E-03 | | | |
| **34 INTERACTION WITH THE ENVIRONMENT** | | | | | | |
| 34.07 cell adhesion | 1.26 | 0.21 | 4.27E-02 | | | |
| 34.07.01 cell-cell adhesion | 1.26 | 0.16 | 2.59E-02 | | | |
| **40 CELL FATE** | | | | 7.03 | 4.45 | 1.90E-02 |
| 40.01 cell growth / morphogenesis | | | | 6.72 | 3.88 | 7.89E-03 |
| **42 BIOGENESIS OF CELLULAR COMPONENTS** | | | | | | |
| 42.04 cytoskeleton/structural proteins | | | | 6.42 | 4.11 | 2.71E-02 |
| 42.10 nucleus | | | | 4.58 | 2.43 | 1.28E-02 |
| 42.10.05 nuclear membrane | | | | 1.83 | 0.48 | 4.40E-03 |
| 42.16 mitochondrion | 7.59 | 2.78 | 1.45E-03 | | | |
| **43 CELL TYPE DIFFERENTIATION** | 12 | 7.37 | 2.27E-02 | | | |
| 43.01 fungal/microorganismic cell type differentiation | 12 | 7.37 | 2.27E-02 | | | |
| 43.01.03 fungal and other eukaryotic cell type differentiation | 12 | 7.37 | 2.27E-02 | | | |
| 43.01.03.09 development of asco- basidio- or zygospore | 6.96 | 2.72 | 3.73E-03 | | | |

Grp occ - number of genes in the cluster belonging to the specified functional category;
Genome occ - number of genes in the genome belonging to the specified functional category.

Amongst the 3 differentially regulated genes after 24 hrs of cold storage, *STE14* was more abundant following an increment in the storage temperature whilst transcripts of *YJL144W* and *HSP10* were present at lower levels. Both *YJL144W* and *HSP10* are stress-responsive genes; whilst the former is induced during osmotic stress and starvation (Roggenkamp *et al.*, 1980, Gasch *et al.*, 2000) the latter is a mitochondrial chaperonin involved in protein folding and sorting (Cronan and Wallace, 1995). None of the microarray entities demonstrated a significant difference in their transcript levels after 48 hrs. As a result, no conclusions could be drawn except that the transcriptional status of *S.pastorianus* W34/70 is seemingly unaffected by an increase in storage temperature after 48 hrs of anaerobic maintenance, at least under the laboratory-scale conditions used in current study.

#### 5.3.2.5 Cold Stress during cropped brewing yeast slurry storage

During slurry storage between successive fermentations, brewing yeast are exposed to low temperature and therefore potentially cold stress. To establish whether lager yeast elicit a cold stress response to storage temperatures, the expression of key cold stress genes by lager strains when exposed to storage conditions was investigated. Expression of 61 cold stress (CS) genes have been either reported (Kandror *et al.*, 2004, Homma *et al.*, 2003, Wolfe and Bryant, 1999, Murata *et al.*, 2006, Kudo *et al.*, 1996, Sahara *et al.*, 2002, Schade *et al.*, 2004, Zhang *et al.*, 2001) or hypothesised (Mewes *et al.*, 1997, Schade *et al.*, 2004) to occur following thermal downshift of haploid laboratory strains. In the current study, 47 out of 61 genes were significantly regulated during slurry storage at 4 °C. Changes in gene expression were deemed significant where a differential regulation of

greater than 10% (FC > 1.1) and $p < 0.05$ (One way ANOVA with BH-FDR correction) had occurred.

**Table 5-9 - List of cold stress responsive genes that were investigated during cold storage of cropped brewing yeast. Gene descriptions were obtained following those of the *Saccharomyces Genome Database* (Hong EL *et al.*, 2007 , Christie *et al.*, 2009, Engel *et al.*, 2010).**

| Gene Symbol | Function |
|---|---|
| ***TIP1*** | Major cell wall mannoprotein |
| ***UPC2/MOX4*** | Sterol regulatory element binding protein |
| ***TIR1*** | Cell wall mannoprotein of the Srp1p/Tip1p family |
| ***TIR2*** | Putative cell wall mannoprotein of the Srp1p/Tip1p |
| ***TIR3*** | Cell wall mannoprotein of the Srp1p/Tip1p family |
| ***TIR4*** | Cell wall mannoprotein of the Srp1p/Tip1p family |
| ***DAN1*** | Cell wall mannoprotein with similarity to TIR protein family |
| ***PAU23/DAN2*** | Cell wall mannoprotein with similarity to TIR protein family |
| ***PAU24/DAN3*** | Cell wall mannoprotein with similarity to TIR protein family |
| ***PAU14///PAU1*** | Member of the seripauperin multigene family |
| ***PAU2*** | Member of the seripauperin multigene family |
| ***PAU20/PAU4*** | Member of the seripauperin multigene family |
| ***PAU5*** | Member of the seripauperin multigene family |
| ***PAU6/PAU18*** | Member of the seripauperin multigene family |
| ***PAU7*** | Member of the seripauperin multigene family |
| ***FBA1/LOT1*** | Fructose 1,6-bisphosphate aldolase |
| ***RPL2B/LOT2*** | Protein component of the large (60S) ribosomal subunit |
| ***NOP1/LOT3*** | Nucleolar protein |
| ***LOT5*** | Protein of unknown function |
| ***LOT6*** | FMN-dependent NAD(P)H:quinone reductase |
| ***TPS1*** | Synthase subunit of T6P synthase/phosphatase complex* |
| ***TPS2*** | Phosphatase subunit of the T6P synthase/phosphatase complex* |
| ***TPS3*** | Regulatory subunit ofT6P synthase/phosphatase complex* |
| ***TSL1*** | Large subunit of T6P synthase/phosphatase complex* |
| ***NTH1*** | Neutral trehalase |
| ***NSR1*** | Nucleolar protein that binds nuclear localization sequences |
| ***OLE1*** | Delta(9) fatty acid desaturase |
| ***MGA2*** | ER membrane protein involved in regulation of OLE1 transcription |
| ***OPI3*** | Phospholipid methyltransferase |
| ***DBP2*** | ATP-dependent RNA helicase of the DEAD-box protein family |
| ***RPA49*** | RNA polymerase I subunit A49 |
| ***GPD1*** | NAD-dependent glycerol-3-phosphate dehydrogenase |
| ***CPR6*** | Peptidyl-prolyl cis-trans isomerase (cyclophilin) |
| ***CPR8*** | Peptidyl-prolyl cis-trans isomerase (cyclophilin) |
| ***ERG10*** | Acetyl-CoA C-acetyltransferase |

| | |
|---|---|
| ***IMH1*** | Protein involved in vesicular transport |
| ***LHS1/CER1*** | Molecular chaperone of the endoplasmic reticulum lumen |
| ***YVH1*** | Protein phosphatase |
| ***TCP1/CCT1*** | Alpha subunit of chaperonin-containing T-complex |
| ***CCT2*** | Subunit beta of the cytosolic chaperonin Cct ring complex |
| ***CCT3*** | Subunit of the cytosolic chaperonin Cct ring complex |
| ***CCT5*** | Subunit of the cytosolic chaperonin Cct ring complex |
| ***CTT1*** | Cytosolic catalase T |
| ***PRX1*** | Mitochondrial peroxiredoxin with thioredoxin peroxidase activity |
| ***GTT2*** | Glutathione S-transferase capable of homodimerization |
| ***BFR2*** | Essential protein that is a component of 90S preribosomes |
| ***MSN2*** | Transcriptional activator related to Msn4p |
| ***MSN4*** | Transcriptional activator related to Msn2p |
| ***HSP 10*** | Mitochondrial matrix co-chaperonin; inhibits Hsp60p ATPase activity |
| ***HSP 12*** | Plasma membrane protein |
| ***Hsp 26*** | Small heat shock protein (sHSP) with chaperone activity |
| ***HSP 30*** | Plasma membrane localized, stress-responsive protein |
| ***HSP 42*** | Small heat shock protein (sHSP) with chaperone activity |
| ***HSP60*** | Tetradecameric mitochondrial chaperonin |
| ***HSP82*** | Hsp90 chaperone |
| ***HSP 104*** | Heat shock protein that cooperates with Hsp40 and Ssa1p |
| ***HSP150*** | O-mannosylated heat shock protein |
| ***SSA1*** | ATPase involved in protein folding and nuclear transport |
| ***SSA2*** | ATP binding protein involved in protein folding and protein import |
| ***SSA3*** | ATPase involved in protein folding and the response to stress |
| ***SSA4*** | Heat shock protein that is highly induced upon stress |

* T6P indicates trehalose-6-phosphate

For the lager strain assessed, most of the cold-responsive genes belonging to the seripauperin gene family demonstrated significant suppression in transcript levels after the first 6 hrs of storage at 4 °C (Figure 5.9A and B). The only exception was *UPC2* (also known as *MOX4*) which was significantly induced (by 1.5 fold) during the initial stages of storage followed by a gradual reduction until 48 hr (Figure 5.9A). The mRNA levels of *PAU2*, *PAU5* and *PAU7* were largely similar to those preceding storage (0 hrs) (Figure 5.9B). In contrast, a group of seripauperin encoding genes including *PAU1/PAU14*, *PAU24*, *PAU4/PAU20*, *PAU6/PAU18* and *DAN1* seemed to be induced by 1.3

to 1.9 fold after 24 hrs of 4 °C storage (Figure 5.9A). This response was sustained to varying degrees after 48 hrs. (1.9 fold; Figure 5.9A). No significant difference in transcript levels of *TIP1* and *TIR1* was observed. Among other *TIP/TIR* gene family members, chronic repression in *TIR4* transcript levels was observed (Figure 5.9B). Following initial decline in transcript abundance, genes responsible for encoding the various subunits of the trehalose-6-phosphate synthase/phosphatase complex (*TPS1, TPS2, TPS3* and *TSL1*) along with neutral trehalase encoding *NTH1* returned to median expression levels after 24hrs of slurry storage (Figure 5.9C). At the end of storage, *TPS1* and *NTH1* demonstrated 1.9 fold and 1.4 fold induction in transcript levels. All the three members of the *LOT* gene family namely *RPL2B* or *LOT2*, *NOP1* or *LOT3* and *LOT5*, were induced by 1.5-1.7 fold in the first 6 hrs of storage at 4 °C (Figure 5.9D). This induction was somewhat transient and remained largely un-sustained with progression of cold incubation.

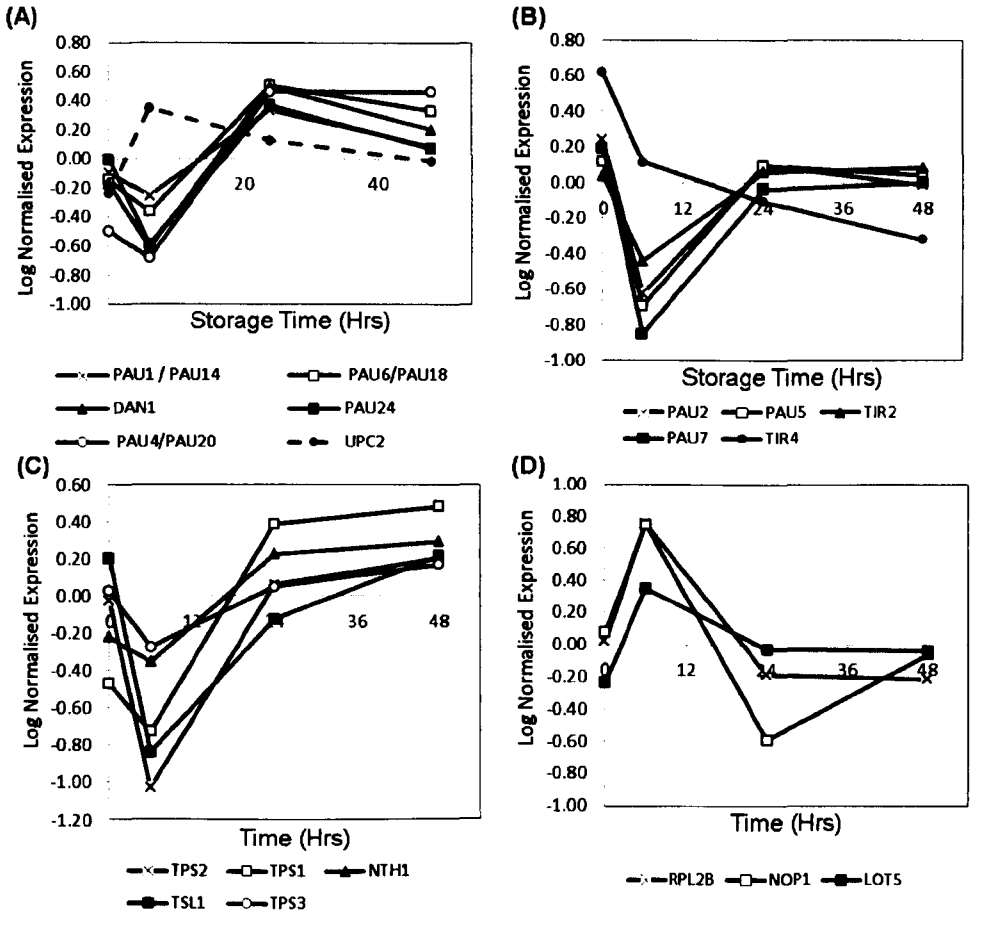



**Figure 5.9 – Transcription profiles of major cold-inducible genes including the seripauperin (A and B), trehalose (C) and LOT (D) gene families during anaerobic storage of cropped yeast at 4 °C. Data points represent the mean of three per-gene normalized transcription values. Only those genes that were differentially expressed (> 1.1 fold change; $p$ <0.05) have been shown.**

Expression of *NSR1*, one of the first reported cold-stress induced genes involved in pre-rRNA processing in *S.cerevisiae* (Kondo *et al.*, 1992), was considerably up-regulated (4 fold) during the first 6 hrs of slurry storage (Figure 5.10A). Henceforth, this trend continued and *NSR1* transcript levels demonstrated the highest magnitude of induction (7 fold) amongst the investigative CS genes after 48 hrs of incubation. Other transcription related genes such as RNA helicase encoding *DBP2* and *RNA* polymerase subunit encoding *RPA49* also demonstrated up-regulation (almost 3 fold) throughout

cold incubation (Figure 5.10A). *YVH1*, whose product is required for a late-step in the maturation of the 60S ribosomal subunit, demonstrated peak expression (2 fold) in the initial storage phase followed by return to median transcript levels (Figure 5.10A). Amongst the genes encoding different subunits of the eukaryotic cytoplasmic chaperonin containing the T-complex polypeptide–1 (CCT), mRNA levels of *CCT2* were consistently augmented (1.3 fold induction after 48 hrs) whilst *TCP1* (or *CCT1*) and *CCT2* transcript levels were continuously down-regulated (Figure 5.10B). Genes involved in detoxification of reactive oxygen species (ROS), such as *CTT1*, *GTT2* and *PRX1*, and known to be induced under both cold and oxidative stress conditions were largely devoid of any obvious increment in expression (Figure 5.10B). Transcript abundance of the bulk of the cold-responsive *HSP*s was considerably suppressed in W34/70 populations and only *HSP12* and *HSP30* demonstrated slight induction in expression following 48 hrs of maintenance at 4 °C (Figure 5.10C and Figure 5.10D).

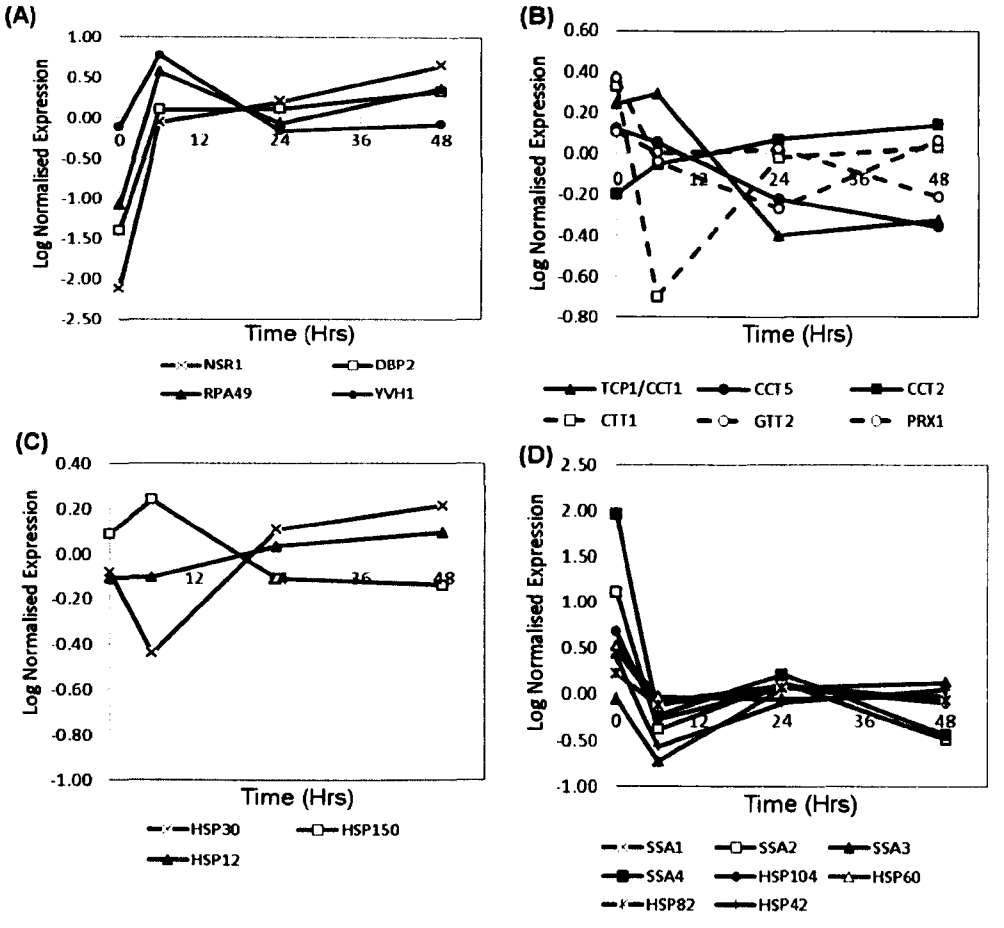



**Figure 5.10 – Transcription profiles of major cold-inducible genes during anaerobic storage of cropped yeast at 4 °C. The selected genes were involved in RNA processing (A), removal of reactive oxygen species (B) and formation of CCT chaperonins (B) and heat shock proteins (C and D). Data points represent the mean of three per-gene normalized transcription values. Only those genes that were differentially expressed (> 1.1 fold change; $p$ <0.05) have been shown.**

*GPD1* and *OPI3* which are known for their association in glycerol and phospholipid biosynthesis demonstrated repression during early storage (Figure 5.11A). Subsequently, a progressive increase in the transcript levels of the former was observed whereas the latter demonstrated peak expression (1.3 fold higher) after 24 hrs of cold storage. *ERG10* and *IMH1*, did not show any obvious induction throughout storage (Figure 5.11A) whilst a marginal but sustained increase in transcript abundance of *GSH1* was observed (Figure

5.11B). mRNA levels of cyclophilin-encoding *CPR8* were 1.3 fold higher after 6 hrs whilst *CPR6* demonstrated chronic repression (Figure 5.11B). Interestingly, certain genes known for their induction during cold stress in *S.cerevisiae* such as the transcription factor encoding *MSN2* and *MSN4* and Δ9-desaturase encoding *OLE1* were not differentially expressed during brewing yeast storage at 4 °C.

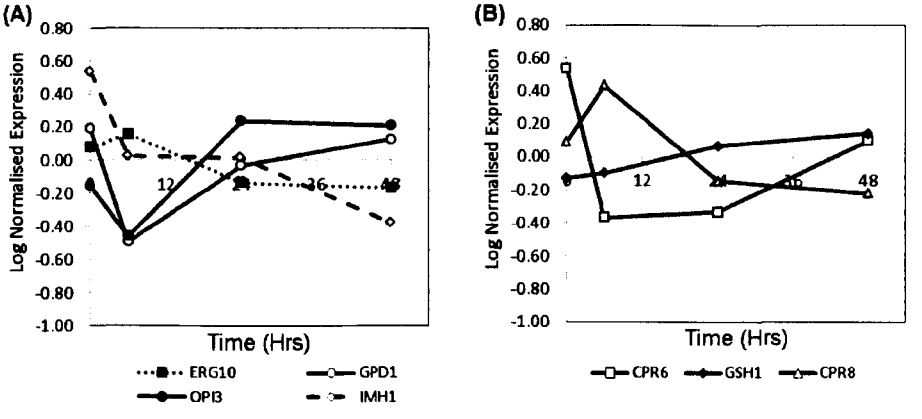



**Figure 5.11 – Transcription profiles of major cold-inducible genes during anaerobic storage of cropped yeast at 4 °C. Chart (A) encompasses genes associated with glycerol and phospholipid biosynthesis whilst (B) includes cyclophilin- and glutathione-encoding genes. Data points represent the mean of three per-gene normalized transcription values. Only those genes that were differentially expressed (> 1.1 fold change; $p$ <0.05) have been shown.**

### 5.3.2.6 Impact of higher cropped yeast storage temperature on expression of cold-inducible genes

The transcript abundance of the 61 cold responsive genes was further monitored following an increase in cropped yeast storage temperature from 4 °C to 10 °C. Following 6 hrs of anaerobic storage, 11 genes demonstrated a significantly different ($p$ < 0.05, fold change of > 10%) expression profile, of which 5 genes demonstrated increased transcription whilst 6 genes were

repressed (Figure 5.12). Despite reduced mRNA levels of the transcription factor encoding *UPC2* (or *MOX4*), transcript levels of *TIR2* and *TIR4* which are inducible by UPC2p were higher following temperature increment from 4 to 10 °C (Figure 5.12). The temperature increment also prompted induction of some of the HSPs (*SSA4, HSP104* and *HSP42*) with concomitant down - regulation (by as much as 4.1 fold) of the transcription related RNA processing genes *RPA49*, *NSR1* and *DBP2* in the early stages of storage (Figure 5.12). mRNA levels of *YVH1* and *MGA2* were also diminished during cropped yeast storage at 10 °C. However, none of these genes were significantly regulated following 24 hrs of storage and *HSP10* was the only differentially transcribed cold responsive gene. Indeed, the lager yeast transcriptome seemed unaffected by an increase in temperature following 48 hrs (Figure 5.12).

**Figure 5.12 - Relative transcript abundance of cold-inducible genes that were differentially expressed during anaerobic storage of cropped brewing yeast slurry at 10C in comparison to storage at 4C. Only genes showing a fold change of > 10% at $p < 0.05$ have been displayed. Red bars indicate up-regulation whilst green bars represent down-regulation.**

| Genes | Fold Change - 10°C vs 4°C | | |
|---|---|---|---|
| | 6 Hrs | 24 Hrs | 48 Hrs |
| *UPC2* | 1.7 | - | - |
| *TIR4* | 1.3 | - | - |
| *TIR2* | 1.5 | - | - |
| *SSA4* | 2.0 | - | - |
| *HSP104* | 1.4 | - | - |
| *HSP42* | 1.3 | - | - |
| *RPA49* | 4.3 | - | - |
| *NSR1* | 2.6 | - | - |
| *DBP2* | 3.6 | - | - |
| *YVH1* | 2.3 | - | - |
| *MGA2* | 1.4 | - | - |
| *HSP10* | - | 1.3 | - |

## 5.4 Discussion

The transcriptional response of yeast following thermal downshift has been previously studied in haploid laboratory strains but not polyploidy or aneutetraploid strains involved in the production of beer. Furthermore the exact molecular mechanism governing cellular survival at low and near freezing temperatures has not yet been elucidated (Aguilera *et al.*, 2007). Although Rodriguez-vargas *et al.* (2002) assessed the cold stress response of exponentially growing industrial baker's yeast strains, no studies concerning brewing yeast have been reported. Low temperatures are not the only environmental condition to which yeast can be exposed during fermentation and cold storage. Anaerobiosis, which typically is applied to slurries during storage, is also known to modulate yeast gene expression (Wu *et al.*, 2001, Tu *et al.*, 1996). The objective of the current study was to establish the impact of cold storage on the transcriptome of lager brewing yeast. To address this, lager yeast was stored at 4 and 10°C and the transcriptional responses were assessed.

### 5.4.1 Impact of anaerobic storage at 4 °C on yeast transcriptome

The transcriptional profile was monitored during 48 hrs of anaerobic slurry storage at 4°C. The response involved the activation of 15% of *S.pastorianus* genome spanning diverse functional categories, as determined by FunCat MIPS (see Section 5.3.2.3.). This suggests that the lager yeast strain, W34/70, elicits a global genomic response during anaerobic cropped slurry storage. The majority of the change in global gene expression occurring within the first six hours of storage was confined to that particular stage (Figure 5.7b). The yeast transcriptome was relatively stable during the latter stages of storage (24 and 48 hr) (Figure 5.7b). The transcriptional response

could thus be delineated into two phases – (i) the early adaptive response (EAdR) focused predominantly on immediate cellular adaptation in the initial phase of storage and (ii) the sustained acclimatory response (SAcR) for cellular acclimatization to long term storage.

The EAdR was characterized by transient induction (predominantly KMeans cluster 2) and repression (mainly KMeans cluster 1) of particular sets of genes (Section 5.3.2.3.1). The bulk of the genes induced during EAdR were associated with RNA processing and *de novo* synthesis of ribosomes through coordinated activation of yeast's transcriptional and translational machineries Table 5-2 and Table 5-3). The deleterious effects of cold shock on cellular ribosomes have been widely reported (Aguilera *et al.*, 2007, Al-Fageeh and Smales, 2006, Sahara *et al.*, 2002, Schade *et al.*, 2004). Mutants with defects in proteins involved in ribosomal proteins (and thus translation) or pre-RNA processing (for ribosome biogenesis and assembly) demonstrated stunted growth at low temperatures (Spellman *et al.*, 1998, Zhang *et al.*, 2001, Tehlivets *et al.*, 2007). Thus the extensive remodelling of the translational machinery following thermal downshift seems crucial for cellular adaptation and subsequent recovery from cold stress. Thermal downshift can compromise translation initiation (Cheng *et al.*, 1995, Rowen *et al.*, 1992) due to increased stabilization of mRNA secondary structures (Thieringer *et al.*, 1998, Schade *et al.*, 2004) and thus compromising the functions of both RNA and DNA polymerases, since it becomes increasingly difficult to unwind nucleic acids. In bacteria, ATP-dependent RNA helicases have been implicated in the removal of secondary mRNA structures (Rowen *et al.*, 1992). Mutations in some of the yeast RNA helicase encoding genes belonging to the DEAD-box protein

family such as *DED1* (Farkas *et al.*, 1991) and *DBP2* (Daran *et al.*, 1997) have also been associated with cold-sensitive phenotypes. In the current chapter, induction of *DBP2, DBP7* and *DBP10* known for encoding RNA helicases belonging to the DEAD-box family was observed during the EAdR. Thus it is possible that RNA helicases such as DBP2p, DBP7p and DBP10p along with other factors involved in RNA-binding and processing might be required for unwinding or removing the cold-stabilized secondary mRNA structures to allow efficient translation initiation.

The SAcR was characterized by a coordinated repression of bulk of the regulated genome (K Means Cluster 3, Figure 5.8). This seemed primarily directed at cellular pathways related to – (i) substrate breakdown (amino acid, urea metabolism) and synthesis (ATP, thiamine and carbohydrate biosynthesis), (ii) intra- and trans-cellular transport ((repression of genes encoding ammonium (*MEP1*, *MEP2*), amino acid (*PUT4, ALP1, MUP1*), hexose- (*HXT2*, *HXT17*), potassium- (*TRK1*) and sulphate (*SUL1*) transporters)) and (iii) inhibition of active cellular growth (downregulation of functions associated with DNA replication, cell cycle events and synthesis/assembly of structural proteins) (Table 5-4 and Table 5-5). Such wide scale repression of genes is reminiscent of a typical genomic response following cold shock (Kudo *et al.*, 1996, Murata *et al.*, 2006, Sahara *et al.*, 2002, Schade *et al.*, 2004) and is perhaps expected in conditions of growth arrest and minimal nutrient supply. Cold-induced reduction in cellular transcriptional and translational efficiency could enforce repression of majority of genes except those whose induction is critical for coping with the stressful environment (Sahara et al, 2002; Schade et al, 2004). Another reason for large-

scale gene repression could be yeast's general tendency to conserve energy under most stressful conditions as part of the activation of the general stress response (Gasch, 2002, Gasch *et al.*, 2000). Nevertheless, decrease in cellular activity following thermal downshift is vital for preserving brewing yeast physiology during storage and helps in maintaining its fermentation potential.

Amongst the genes whose up-regulation was necessitated during the SAcR (K Means Cluster 4, Figure 5.8) were those relating to the cellular transcriptional machinery, particularly rRNA processing (Table 5-6). Interestingly, this result is in contrast to an earlier report (Murata *et al.* 2006) where abrupt down regulation of most of the transcription and ribosomal genes was observed following extended incubation at 4°C. This was primarily attributed to a low requirement of protein synthesis at low temperatures due to minimal cell growth and consequently no obvious need for modulating transcriptional machinery after initial adaptation (Murata *et al.*, 2006) (Aguilera *et al.*, 2007). Whilst most of the differentially expressed cell cycle- and DNA synthesis-related ORFs were also repressed during the SAcR thereby indicating a lack of cell division, continuous emphasis on regulation of the yeast transcriptional machinery during both EAdR and SAcR suggests that this reorganisation might be crucial for cell survival during brewing yeast slurry storage. It is noteworthy that low temperature is not the sole stress to which are exposed during brewing yeast storage and genes associated with nutrient starvation (see Table 5-6) and anaerobiosis were also differentially expressed during SAcR. Thus, it is tempting to speculate that simultaneous occurrence of multiple stresses during cropped yeast storage might be responsible for sustained cellular need to modulate its transcriptional machinery. Further work

is needed to ascertain this hypothesis and to examine whether simultaneous application of different environmental stresses can have a synergistic effect upon yeast behaviour during slurry storage.

### 5.4.2 Impact of higher storage temperature on yeast transcriptome

More than 99% of the temperature-dependent changes in cropped yeast gene expression occurred in the early storage phase (Table 5-7) suggesting that the sensitivity of yeast transcriptome to temperature increment is perhaps limited to the initial storage stages. A surge in cellular energy demand was observed during EAdR following temperature increment from 4 to 10 °C (Table 5-8). This is probably due to the ability of lager brewing yeast to grow and thus conserve its metabolic functions between 8 – 12 °C on account of its hybrid nature (Dunn and Sherlock, 2008, Nakao *et al.*, 2009, Sato *et al.*, 2002). Increasing the storage temperature from 4 to 10 °C also seemed to enhance yeast stress response during early storage particularly the expression of genes related to oxidative and osmotic tress (Table 5-8). This is in contrast to previous reports that suggest induction of antioxidant genes and mild oxidative stress following temperature downshift to low temperatures (Huang *et al.*, 1998) which becomes progressively intense towards near-freezing conditions (Aguilera *et al.* 2007). The reason for this phenomenon is not yet known and warrants further investigation. Higher storage temperature also lead to a reduction in cellular efforts towards refurbishment of its transcriptional machinery thus indicating that cellular transcription is compromised to a lower extent during slurry storage at higher temperatures (Table 5-8).

### 5.4.3 Evidence for increased cold stress at the near-freezing temperature

The cold stress biomarker, *NSR1* was differentially regulated during W34/70 slurry storage at both near-freezing (4 °C) and cold (10 °C) temperatures. *NSR1* transcript levels were continuously upregulated during 4°C-storage and demonstrated maximum induction amongst the CS genes (Figure 5.10A) suggesting that increased NSR1p might be critical for cropped yeast survival at 4°C. Indeed, following thermal downshift *NSR1* deletion mutants have displayed severely impaired pre-rRNA processing and consequently stunted growth (Kondo *et al*, 1992). Assuming that *NSR1* induction is an unequivocal manifestation of cold stress in yeast, *NSR1* repression during EAdR following storage temperature increment from 4 to 10 °C (Figure 5.12) is representative of enhanced cold stress during the initial stages of cropped slurry storage at 4 °C. Other cellular-transcription associated CS genes, such as *DBP2* and *RPA49* (Sahara *et al.*, 2002, Schade *et al.*, 2004), also demonstrated regulation trends similar to *NSR1* (Figure 5.10A and Figure 5.12) and provides further evidence of increased cold stress at 4 °C.

In the model haploid laboratory strains of the species *S.cerevisiae*, the transcriptional activator encoding *MGA2* has been postulated to act as a cold sensor (Nakagawa *et al.*, 2002). Reduced *MGA2* mRNA in cropped yeast stored at 10 °C (Figure 5.12) thus gives further indication of the occurrence of increased cold stress in the initial stages of cropped yeast storage at 4 °C. *YVH1*, which encodes for a dual-specificity protein phosphatase, is induced at low temperatures (Schuller *et al.*, 1992) and is even involved in cold growth (Hoja *et al.*, 2004) probably through cold-shock signalling via the cAMP-PKA

pathway (Torkko *et al.*, 2001, Aguilera *et al.*, 2007). Diminished *YVH1* transcript abundance at the higher storage temperature (Figure 5.12) thus suggests increased cAMP-PKA activity at the near-freezing temperature probably due to elevated cold stress.

### 5.4.4 Impact of anaerobiosis on CS gene transcription

Despite being widely accepted as characteristic cold-stress biomarkers in yeast (Schade *et al.*, 2004) (Kowalski *et al.*, 1995) no significant difference in *TIP1*, *TIR1* or *OLE1* transcript levels was observed at any of the assessed storage temperatures. Utilizing a fermentation regime similar to that of G0F, significant anaerobiosis-induced activation of these genes was observed during the latter fermentation stages (Ashraf, 2012). Thus it is tempting to speculate that the presumably high *TIP1, TIR1* and *OLE1* transcript abundance at the end of G0F might suffice cellular needs following temperature downshift. Indeed, such lack of *TIP1* and *TIR1* induction on account of continued anaerobiosis has also been reported by Tai and co-workers (2001) in cold-shocked chemostat cultures. Amongst other *TIR* genes, increased *TIR2* and *TIR4* mRNA levels following change of storage temperature from 4 to 10 °C (Figure 5.12) might represent cellular attempt at anaerobic growth. Thus, although the *TIP/TIR* genes are influenced by both cold shock and anaerobiosis (Donzeau *et al.*, 1996, Abramova *et al.*, 2001a), it is perhaps the latter that dictates their transcription during conditions of cropped yeast storage prevalent in the brewery.

Increased transcript abundance of PAU genes (particularly *PAU4/PAU20*) and *DAN1* was evident during yeast storage at both 4°C and 10

°C (Figure 5.9A). Although, DAN1 induction could be attributed to the occurrence of anaerobiosis during slurry storage (Cohen *et al.*, 2001), the exact reason for activation of PAU genes could not be ascertained due to their propensity to be regulated by both cold-stress (Murata *et al.*, 2006) and anoxia (Donzeau *et al.*, 1996, Abramova *et al.*, 2001a). Nevertheless, the activation of these mannoprotein-encoding genes seems to be required for cellular survival during brewing yeast slurry storage. Two of the LOT genes namely *RPL2B* (or *LOT2*) and *NOP1* (or *LOT3*) are involved in ribosome biogenesis (Zhang *et al.*, 2001) and their induction during the EAdR at both storage temperatures (Figure 5.9D) highlights cellular efforts to repair the bottlenecks related to cellular translation and RNA processing.

### 5.4.5 Environmental Stress Response during cropped slurry cold storage

The environmental stress pathway mediated by the MSN2p and MSN4p transcription factors are known to become activated at lower temperatures (Kandror *et al.*, 2004). Although differential expression of *MSN2* and *MSN4* remained undetected in current chapter, significant regulation of several genes including *TPS1, HSP12, HSP42, SSA4* and *CTT1* which are strictly under the control of MSN2/4p was observed (Aguilera *et al.*, 2007) (Figure 5.9C and Figure 5.10B and D) at both storage temperatures. The activation of *HSP12* (Figure 5.10C), albeit mild, at both 4 and 10 °C conforms to previous observations (Kandror *et al.*, 2004, Gibson *et al.*, 2007, Spellman *et al.*, 1998, Cheng *et al.*, 1995) and HSP12p is probably involved in freezing tolerance and maintenance of membrane organisation by localization in the plasma membrane (Heinisch *et al.*, 1998). Another heat shock protein, HSP30p has been associated with yeast exposure to near-freezing temperatures (Murata *et*

*al.*, 2006). However, it's reported repression at slightly higher temperatures (i.e. 10C) (Sahara *et al.*, 2002) is in disagreement with the findings of current study (Figure 5.10C) presumably due to strain and experimental differences. *HSP30* is induced in glucose-limited conditions due to its role in energy conservation by repressing plasma membrane H+-ATPase activity and limiting excessive ATP consumption (Walker *et al.*, 1991). Increased transcript abundance of a subset of heat shock proteins namely HSP104p, HSP42p and SSA4p during the EAdR at the increased storage temperature (Figure 5.12) is probably directed at assisting protein folding and removal of protein aggregates. Both *HSP42* and *SSA4* are reportedly induced during starvation (Albertyn *et al.*, 1994c) (Albertyn *et al.*, 1994b) and marginally higher transcript abundance of these *HSP* genes could also indicate enhanced yeast starvation during early storage at the higher temperature.

## 5.5 Conclusion

In this chapter, the transcriptional response of *S.pastorianus W34/70* during cropped yeast storage has been evaluated for the first time. Based on the available genetic evidence, it seemed that traditional storage regimes that employed near-freezing temperatures (4 °C) might exert increased cold stress on brewing yeast. On the other hand, application of higher storage temperatures can impose other unfavourable conditions. Surprisingly, both kinds of stress responses were absent in the subsequent acclimatory phase of storage. One possible explanation could be derived from the hypothesis proposed by Gasch (2002) who suggested that alteration in external environment is accompanied by immediate dramatic regulation of the yeast transcriptome. As the cell gets accustomed to its new surroundings a steady-state is achieved in the gene expression program necessitating only slight modifications in the transcriptome for survival. In this chapter, gene expression analysis considered a global genomic view of brewing yeast behaviour under the two storage regimes and provided indications that the application of higher storage temperature is not necessarily immensely deleterious to the brewing yeast transcriptome. However, a stable transcriptome does not necessarily equate to a lack of change in cellular physiology and thus the impact of change in storage temperature on yeast physiology was also verified (described in Chapter 6).

The lack of regulation of the widely accepted cold-stress biomarkers *TIP1, TIR1, TIR2* and *OLE1* in the current study highlights the ambiguity in their accepted status. It also magnifies the complexities of yeast response in extreme environments that are routinely applied to microorganisms during

industrial operations. From a brewer's point of view, *NSR1* induction during yeast slurry cold storage seems to be a good indicator of the occurrence of cold stress in brewing yeast. However, the current study was limited to a single lager strain and further investigations with different strains need to be conducted before arriving at a definitive conclusion.

# CHAPTER 6: IMPACT OF STORAGE TEMPERATURE ON FERMENTED YEAST SLURRY

## 6.1 Introduction

"Serial repitching" in the brewing industry involves removal of yeast biomass at the end of fermentation (cropping), storage of the harvested (cropped) yeast and re-inoculation into wort (pitching) for successive fermentations. During large scale operations, storage of the cropped yeast is necessary to permit fermenter cleaning and provide flexibility in the supply chain of wort which the yeast is required to ferment. As alluded to before (Section 1.4.5, Chapter 2), cropped yeast is usually stored either in the form of pressed cake or as a slurry (Boulton and Quain, 2001, Briggs *et al.*, 2004, Quain and Tubb, 1982). Storage as pressed cake is usually associated with traditional top-cropped fermenters and despite its economic superiority this mode of storage has been suggested to exert an adverse effect upon yeast quality. This is primarily due to yeast exposure to atmospheric air rendering it more prone to infections, compromised intracellular glycogen content, difficulty in temperature control and probable heterogeneity in microbial population distribution (Boulton and Quain, 2001, Quain and Tubb, 1982). As a result the majority of breweries tend to store pitching yeast as a slurry i.e. a suspension of yeast in entrained beer taken directly from the fermenter. Typically cropped yeast slurry storage is undertaken in a closed vessel (preferably under an anaerobic atmosphere) (Quain and Tubb, 1982, Boulton and Quain, 2001) and facilitates aseptic biomass handling, facilitates transport via pumping and homogenous attemperation of microbial populations.

As alluded to before (Section 4.1,Chapter 4), brewing yeast quality during storage is influenced by a number of factors including; the duration of storage (McCaig and Bendiak, 1985a) (McCaig and Bendiak, 1985b); application of oxygenation (Verbelen *et al.*, 2009b); rate of agitation (McCaig and Bendiak, 1985a);the application of $CO_2$ to modify the gas environment; nutrient availability (Smart *et al.*, 1999) ; and temperature (McCaig and Bendiak, 1985b). Of these, temperature arguably plays the most important role since it has a direct influence on the metabolic activity of the yeast during storage. In the presence of a non-fermentable medium, such as ethanol, inappropriate increments in storage temperature can accelerate the mobilization of stored carbohydrate reserves forcing the yeast population to degrade vital cellular components (Quain and Tubb, 1982) leading to autolysis and cell death. Another phenomenon that can affect brewing yeast quality at higher temperature storage temperatures is "autofermentation" whereby reserve carbohydrates are fermented to generate ethanol and carbon dioxide (Boulton and Quain, 2001, McCaig and Bendiak, 1985b). Autofermentation also results in a substantial loss in slurry viability. Non-optimal storage temperatures can therefore result in impaired fermentations causing disruption in supply chain efficiency and escalation of production costs.

As discussed in Chapter 4, storage of propagated brewing yeast slurry at 4 and 10 °C did not seem to elicit notable differences in cellular physiology. However, yeast slurries cropped from fermenter will have experienced a different environment than those harvested from propagation and therefore may exhibit differences in responses to the stresses imposed by cold storage. The process conditions of propagation and fermentation differ with respect to:

process temperature (higher in propagation when compared to fermentation), gas environment (continuous aeration during propagation and predominantly $CO_2$ induced anaerobic conditions during fermentation), exposure to ethanol (for fermentation), low pH (for fermentation) and nutrient starvation (both conditions) (Boulton and Quain, 2001, Gibson *et al.*, 2010, Gibson *et al.*, 2007, Miller *et al.*, 2012). It was therefore deemed important to assess the impact of storage temperature on fermentation derived yeast slurries (G1 slurries). Assessment of the global transcriptome of cropped W34/70 slurry as a function of storage temperature (Chapter 5) suggested that storage at slightly elevated temperatures may not cause adverse phenotypes in lager brewing yeast. To test this hypothesis, key performance indicators were assessed during storage at each temperature and this analysis is the subject of the current chapter.

## 6.2 Results

To address the hypothesis that storage at slightly elevated temperatures would not adversely affect fermentation performance, cropped W34/70 slurry samples stored in 2-port storage vessels (Section 2.10.3, Chapter 2) at 4 and 10 °C were harvested at various intervals (0, 6, 12, 24, 36, 48 and 72 hrs) and stored for subsequent analysis. The samples were analysed for cell viability using citrate methylene violet (Section 2.11.1, Chapter 2), budding index (Section 2.11.3, Chapter 2), glycogen (Section 2.13, Chapter 2), trehalose (Section 2.13, Chapter 2), glycerol (Section 2.15, Chapter 2) and fatty acid distribution (Section 2.14, Chapter 2). The total cellular RNA isolate was labelled and hybridized to GeneChip Yeast Genome 2.0 Array (Affymetrix) (as described in Section 2.16.2, Chapter 2) to investigate the transcript abundance of genes associated with key performance indicator phenotypes.

### 6.2.1.1 Changes in cell viability and budding index during storage

Cropped brewing yeast cell viability was consistently above 95% over 72 hours of anaerobic storage at both 4 and 10°C (see Figure 6.1). Budding Index (BI) was consistently less than 2% at both temperatures (Figure 6.1) indicating a lack of active cell division during slurry storage presumably due to oxygen and nutrient limitation (Miller *et al.*, 2012) (Boulton and Quain, 2001).

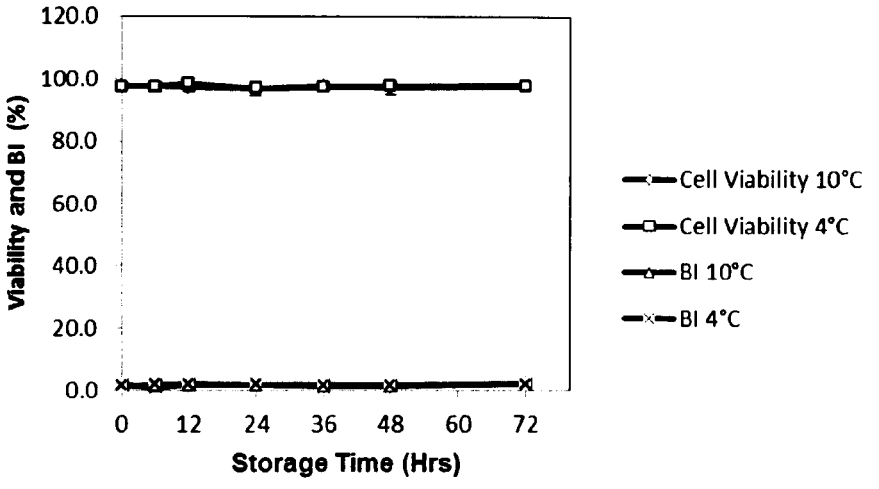



**Figure 6.1 – Cell viability and budding index (BI) during anaerobic W34/70 slurry storage. Yeast populations were maintained anaerobically at 4°C and 10 °C for 72 hours in the absence of agitation. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

### 6.2.1.2 Changes in yeast glycogen during storage

Figure 6.2 demonstrates the change in cellular glycogen content over 72 hours of anaerobic storage at 10 and 4°C. Despite the observation that higher glycogen levels were exhibited by yeast populations stored at 4 °C when compared to those stored at 10 °C, the observed difference was not uniformly statistically significant. During the first few hours of cold storage (after 6 hours) significantly elevated glycogen levels ($p < 0.05$) were observed for *W34/70* slurry stored at 4°C when compared to that stored at higher temperatures. However, this initial observation was not maintained during storage for longer periods of time. This result is important since it is generally accepted in brewing that slurries should not be stored for more than 48 hours and this timescale is usually deemed "best practice". Irrespective of the

temperature deployed glycogen breakdown occurred as a function of duration of storage.

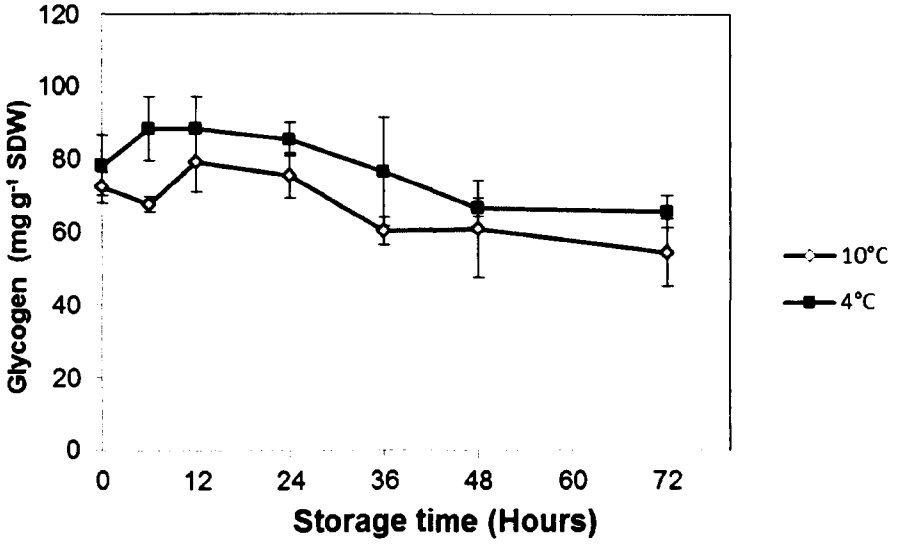



**Figure 6.2 - Intracellular glycogen of *W34/70* during anaerobic yeast slurry storage at 10 °C and 4 °C for 72 hrs in the absence of agitation. Final values have been represented as mg glucose released per gram of slurry dry weight (SDW). Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

A total of 26 genes encode proteins which are known for their involvement in the biosynthesis (GO 0005978), catabolism (GO0005980) and metabolism (GO0005977) of yeast glycogen (Table 6-1). Of these, 15 genes demonstrated a significant ($p < 0.05$ with Benjamini Hochberg FDR correction; fold change > 1.3) difference in transcript levels during slurry maintenance at 4 °C.

**Table 6-1 – List of genes involved in the synthesis (GO0005978), catabolism (GO0005980) and metabolism (GO0005977) of glycogen. Descriptions for gene ontologies (GO) and gene functions were obtained following those of the *Saccharomyces* Genome Database (Hong EL *et al.*, 2007 , Christie *et al.*, 2009, Engel *et al.*, 2010).(Cheng *et al.*, 1995)**

| Gene | Description |
|---|---|
| *GAC1* | Regulatory subunit for Glc7p type-1 protein phosphatase (PP1) |
| *GLC3* | Glycogen branching enzyme |
| *GLG1* | Self-glucosylating initiator of glycogen synthesis |
| *GLG2* | Self-glucosylating initiator of glycogen synthesis |
| *GSY1* | Glycogen synthase with similarity to Gsy2p |
| *GSY2* | Glycogen synthase, similar to Gsy1p |
| *PGM1* | Phosphoglucomutase; converts G1P to G6P |
| *PGM2* | Phosphoglucomutase; converts G1P to G6P |
| *UGP1* | UDP-glucose pyrophosphorylase (UGPase) |
| *GDB1* | Glycogen debranching enzyme |
| *GPH1* | Non-essential glycogen phosphorylase |
| *SGA1* | Intracellular sporulation-specific glucoamylase |
| *AAP1* | Arginine/alanine aminopeptidase, stimulates glycogen accumulation |
| *BMH1* | 14-3-3 protein, major isoform; controls proteome at post-transcriptional level |
| *BMH2* | 14-3-3 protein, minor isoform; controls proteome at post-transcriptional level |
| *GIP2* | Putative regulatory subunit of the protein phosphatase Glc7p |
| *GLC7* | Type 1 serine/threonine protein phosphatase catalytic subunit |
| *GLC8* | Regulatory subunit of protein phosphatase 1 (Glc7p) |
| *PCL10* | Pho85p cyclin |
| *PCL6* | Pho85p cyclin of the Pho80p subfamily |
| *PCL7* | Pho85p cyclin of the Pho80p subfamily |
| *PCL8* | Cyclin, interacts with Pho85p cyclin-dependent kinase |
| *PPG1* | Putative serine/threonine protein phosphatase of the type 2A |
| *REG1* | Regulatory subunit of type 1 protein phosphatase Glc7p |
| *SHP1* | UBX (ubiquitin regulatory X) domain-containing protein that regulates Glc7p phosphatase activity |
| *YPI1* | Inhibitor of the type I protein phosphatase Glc7p |

Six of the ten glycogen synthetic genes (GO0005978) showed a significant change in expression during traditional brewing yeast slurry storage. These included (i) *GLG2*, which (along with *GLG1*) encodes the self-

glucosylating enzyme glycogenin glucosyltransferase that initiates glycogen formation, (ii) *GSY1* and *GSY2* (dominant gene homolog), which encodes glycogen synthases responsible for glycogen chain elongation and (iii) *GLC3* which encodes the glycogen branching enzyme GLC3p (Cheng *et al.*, 1995, Rowen *et al.*, 1992, Farkas *et al.*, 1991). The majority of the differentially expressed genes were down-regulated after the first 6 hrs of slurry storage at 4 °C whilst transcript abundance in the latter stages was more varied (Figure 6.3A and Figure 6.3B). Amongst the glycogen synthesizing genes, *GLC3*, *GSY1* and *GSY2* were up-regulated after 24 hr (Figure 6.3A) whilst a progressive decrease in the transcript abundance of *GLG2* was observed throughout 48 hr of cropped slurry storage at 4 °C (Figure 6.3B). Out of the three known genes associated with glycogen breakdown in *S.cerevisiae* (*GPH1*, *GDB1* and *SGA1*; GO0005980), only glycogen phosphorylase encoding *GPH1* demonstrated a significant change in expression. Following initial down-regulation, increased *GPH1* transcript abundance was observed with peak levels obtained after 48 hrs of slurry storage (Figure 6.3A). Based on the observed transcriptional profiles, the cellular emphasis seemed to be concentrated towards both glycogen accumulation and mobilization with genes involved in both processes being induced during slurry storage. Other differentially expressed glycogen-synthesizing genes included *PGM2* and *UGP1* which encode enzymes catalysing the inter-conversion of glucose-1-phopspahate (G-1-P) to glucose-6-phospahte (G-6-P) and G-6-P to UDP-glucose (UDPG) respectively (Daran *et al.*, 1997). Both these enzymes are vital since they connect glycogen metabolism to the intermediate compounds of the central glucose metabolism. After initial down-regulation, *PGM2* was

slightly induced in a sustained manner until 48 hrs whilst transcription of *UGP1* remained unchanged (Figure 6.3B).

Apart from the above mentioned genes which encode proteins directly associated with glycogen formation or degradation, glycogen metabolism is strictly regulated through several other factors. In yeast, the activity of glycogen synthase (GSY2p) and glycogen phosphorylase (GPH1p) is antagonistically controlled by their phosphorylation state (Huang *et al.*, 1998, Hwang *et al.*, 1989, Parrou *et al.*, 1999, Francois and Parrou, 2001). Dephosphorylation mediated by cellular phosphatases activates GSY2p favouring glycogen accumulation whilst kinase mediated phosphorylation inactivates GSY2p (Francois and Parrou, 2001, Huang *et al.*, 1998, Wu *et al.*, 2001). Four genes, *GIP2*, *GLC8*, *REG1* and *SHP1*, which encode elements involved in regulating the activity of type 1 serine/threonine protein phosphatase (PP1) (encoded by GLC7) (Tu *et al.*, 1996, Zhang *et al.*, 1995, Dombek *et al.*, 1999) were differentially expressed during W34/70 storage at 4 °C (Figure 6.3C). Of these, only *REG1* demonstrated gradual induction during storage whilst transcript levels for the rest were below starting levels for the entire storage duration (Figure 6.3C). *PPG1* which encodes another class of serine/threonine protein phosphatase PP2A (Posas *et al.*, 1993) was induced throughout storage at 4 °C with peak levels after 24 hrs (Figure 6.3D). Both *PCL7* and *PCL10*, who have been suggested to partner the cyclin dependent kinase PHO85p in phosphorylation and thereby inactivation of glycogen synthase (Hoja *et al.*, 2004, Wilson *et al.*, 2010, Torkko *et al.*, 2001) were significantly expressed. Whilst *PCL10* demonstrated peak induction in the initial storage stages followed by gradual down-regulation, *PCL7* was down

regulated throughout the 48 hrs of slurry storage at 4 °C (Figure 6.3D). Simultaneous activation of both protein phosphatase and cellular kinase encoding genes suggests that the phosphorylative state and thus the activity of the glycogen-associated enzymes seem to be tightly controlled at the transcriptional level during cropped *W34/70* storage.

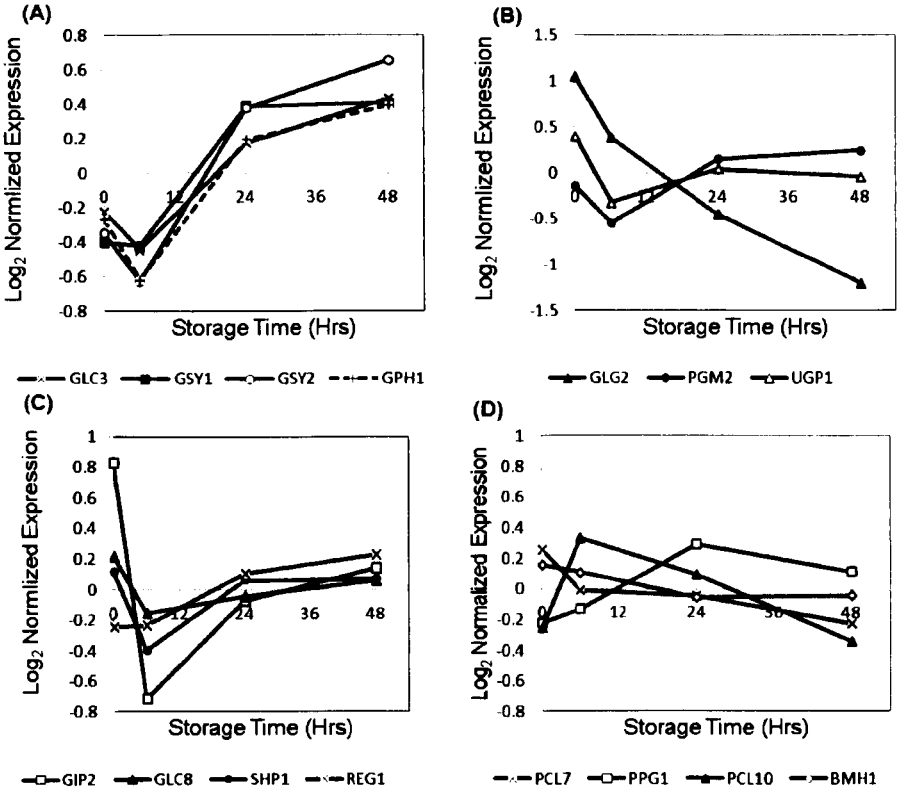



**Figure 6.3 - Transcription profiles of genes involved in the biosynthesis, catabolism (A, B) and metabolism (C,D) of glycogen during W34/70 cropped yeast slurry storage at 4 °C. All presented genes show a statistically significant change in expression of $p < 0.01$ (one-way ANOVA with BH-FDR correction) and fold change of > 1.3. Data points represent the mean of three per-gene normalized transcription values.**

Expression of genes involved in yeast glycogen metabolism was evaluated at the higher storage temperature. This expression change was deemed significant following a fold change of $\geq$ 10% with a statistical significance of $p < 0.05$ (One Way ANOVA with BH-FDR). All of the differences in the transcript abundance of these ORFs observed were confined to the initial storage stages. Five (out of 26) genes differed significantly with respect to transcription between the two storage conditions following 6 hrs of cold incubation (Table 6-2). Two of the differentially expressed glycogen biosynthesis genes (*GLG2* and *UGP1*) were upregulated following 6 hrs of incubation at the higher temperature whereas none of the three glycogen catabolism genes were influenced by the storage temperatures applied in the current study. Amongst the ORFs whose products regulate glycogen metabolism, genes involved in formation of protein phosphatase I (PP1) regulatory subunits (*GAC1*, *GIP2*, *GLC8)* demonstrated comparative enhancement in transcript abundance at 10 °C whilst none of the investigated kinase-encoding genes were induced (Table 6-2). Both of the above observations together suggest partial remodelling of W34/70 metabolome to facilitate glycogen accumulation following an increase in storage temperature. However the absence of such changes in the latter stages of cold storage (24 and 48 hrs) leads to the presence of similar amounts of glycogen in both sets of W34/70 populations (Table 6-2).

| Genes | Fold Change - 10°C vs 4°C | | | |
|---|---|---|---|---|
| | 6 Hrs | | 24 Hrs | 48 Hrs |
| *GIP2* | 1.7 | | - | - |
| *GLG2* | 1.7 | | - | - |
| *UGP1* | 1.2 | | - | - |
| *GLC8* | 1.2 | | - | - |
| *GAC1* | 1.3 | | - | - |

**Table 6-2 - Fold change in the levels of glycogen metabolism genes in *S.pastorianus W34/70* during 48 hrs of anaerobic slurry maintenance at 10 and 4 °C. All genes presented show a statistically significant change in expression ($p < 0.01$) and a fold change cut off of > 1.1 (10%). Red bars indicate up-regulation whilst green bars represent down regulated genes. Data points represent the mean of three per-gene normalized transcription values.**

### 6.2.1.3 Changes in yeast trehalose during storage

Intracellular levels of the disaccharide trehalose were largely independent of both time and temperature of storage until the final 24 hours of cold storage (Figure 6.4). After 36 hours, increased trehalose levels ($p < 0.05$) were observed in brewing yeast stored at 4°C but not for that stored at 10°C. The higher levels observed for the former were largely sustained throughout the period of storage assessed. G1 yeast obtained from 4°C storage had significantly ($p < 0.05$) higher trehalose compared to yeast stored at 10 °C. Final levels indicated a 14% increase in trehalose content under traditional slurry storage regime as opposed to a 9% drop at the higher storage temperature. The impact of this on subsequent fermentation performance is not known.

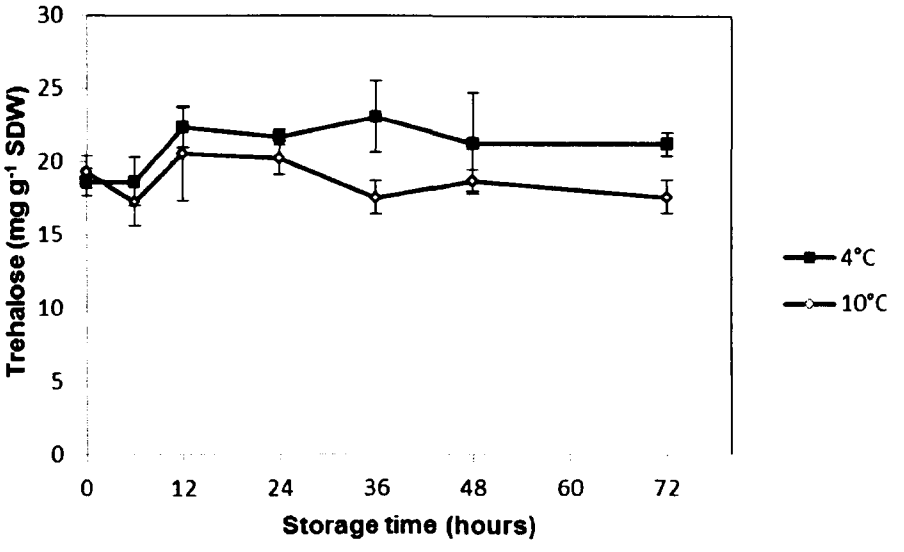



**Figure 6.4 - Intracellular trehalose content of W37/70 during anaerobic storage at 10°C and 4°C over 72 hours without any agitation. Final values have been represented as mg glucose released per gram of slurry dry weight (SDW). Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

Transcription profiles of 12 genes involved in trehalose biosynthesis (GO0005992), catabolism (GO0005993) and associated processes (*MAL 11*, *PGM3*) were monitored during yeast slurry storage at the two temperatures. The genes and their functions have been summarised in Table 6-3. Expression of 8 out of the 12 genes was altered during *W34/70* slurry maintenance at 4 °C. Indeed in a similar manner to that determined for glycogen metabolism, most of the genes involved in trehalose metabolism were suppressed in the first 6 hrs of 4°C-storage (Figure 6.5A). Significant induction of *TPS1* whose protein product catalyses the conversion of glucose-6-phosphate (G6P) and uridine diphosphate glucose (UDPG) into trehalose-6-phosphate was observed after 24 hrs yeast storage (Bell *et al.*, 1992, Francois and Parrou, 2001, Parrou *et al.*,

1997) (Figure 6.5A). Final *TPS1* transcript levels were 1.9 fold higher than those preceding storage. Following initial down regulation, transcript abundance of genes encoding the remaining subunits of the trehalose-6-phosphate synthase complex *TPS2*, *TPS3* and *TSL1* returned to starting transcript levels and then remained unchanged until 48 hrs of storage (Devirgilio *et al.*, 1993, Bell *et al.*, 1998) (Figure 6.5A). Products of *PGM2/3* and *UGP1* catalyze the sequential conversion of G6P to glucose-1-phosphate (G1P) and UDPG respectively thereby providing the necessary substrates for trehalose synthesis (Daran *et al.*, 1997). *PGM3* was considerably induced until 48 hrs and demonstrated the highest fold change (2.3 fold) in the cohort of genes associated with trehalose metabolism (Figure 6.5A). Cytosolic neutral trehalase encoding *NTH1* (Zou *et al.*, 2003) and *NTH2* were significantly induced following 24 hrs of *W34/70* incubation at 4 °C whilst transcript abundance of the vacuolar acid trehalase-encoded by *ATH1* (Johnson *et al.*, 1994) (Hettema *et al.*, 1996) remained largely stable until 48 hrs (Figure 6.5B). *MAL11*, which has been suggested to encode for a transporter protein that enables uptake of extracellular trehalose (Plourde-Owobi *et al.*, 1999) demonstrated no change in regulation throughout cropped yeast storage (data not shown).

None of the assessed genes associated with trehalose metabolism were differentially regulated following an increase in storage temperature from 4 °C to 10 °C.

**Table 6-3 – List of genes associated with trehalose biosynthesis (GO0005992), catabolism (GO0005993) and associated processes. Descriptions for gene ontologies (GO) and gene functions were obtained following those of the *Saccharomyces* Genome Database (SGD). (Hong EL *et al.*, 2007 , Engel *et al.*, 2010, Christie *et al.*, 2009).**

| Gene | Description |
|---|---|
| ***TPS1*** | Synthase subunit of T-6-P complex |
| ***TPS2*** | Phosphatase subunit of T-6-P complex |
| ***TPS3*** | Regulatory subunit of T-6-P complex |
| ***TSL1*** | Large subunit of T-6-P complex |
| ***UGP1*** | UDP-glucose pyrophosphorylase (UGPase) |
| ***PGM1*** | Phosphoglucomutase, converts G-1-P to G-6-P |
| ***PGM2*** | Phosphoglucomutase converts G-1-P to G-6-P |
| ***PGM3*** | Phosphoglucomutase, converts G-1-P to G-6-P |
| ***ATH1*** | Acid trehalase |
| ***NTH1*** | Neutral trehalase, degrades trehalose |
| ***NTH2*** | Putative neutral trehalase |
| ***Mal11/AGT1*** | Inducible high-affinity maltose transporter |

T-6-P complex: trehalose-6-phosphate synthase/phosphatase complex

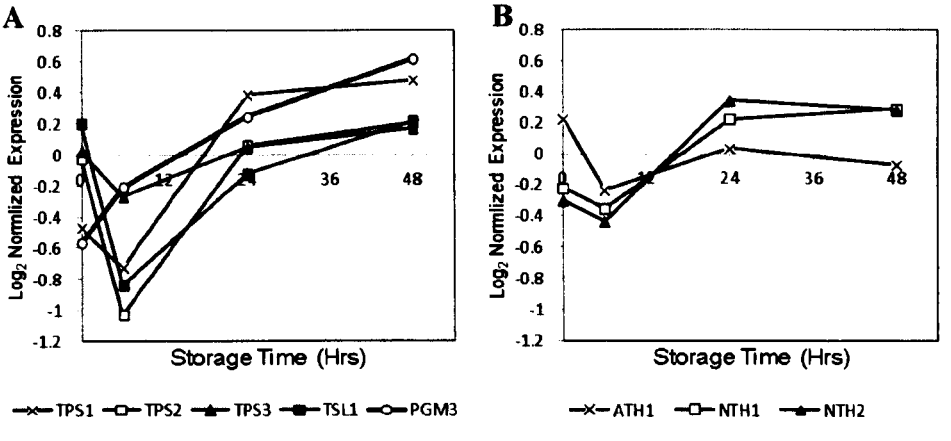



**Figure 6.5 - Transcription profiles of genes associated with the biosynthesis (A) and catabolism (B) of trehalose during W34/70 fermented yeast slurry storage at 4 °C. All presented genes show a statistically significant change in expression of $p < 0.01$ (one-way ANOVA with BH-FDR correction) and fold change of > 1.3. Data points represent the mean of three per-gene normalized transcription values.**

### 6.2.1.4 Changes in intracellular glycerol during storage

Intracellular glycerol content in *W34/70* cell populations stored at 4 °C remained largely unchanged until 48 hours of storage (refer to Figure 6.6). Final levels after 72 hrs of storage were almost 40% higher in comparison to the start of cold-storage (1.5 ± 0.1 at 0 hrs to 2.1 ± 0.2 after 72 hrs). An increase in storage temperature to 10 °C, resulted in a significantly higher glycerol content ($p < 0.05$) after 12 hours of incubation followed by gradual dissimilation and return to basal levels after 36 hrs with no change thereafter. After 72 hrs of incubation glycerol content was significantly elevated in populations maintained at 4 °C in comparison to storage at 10 °C.

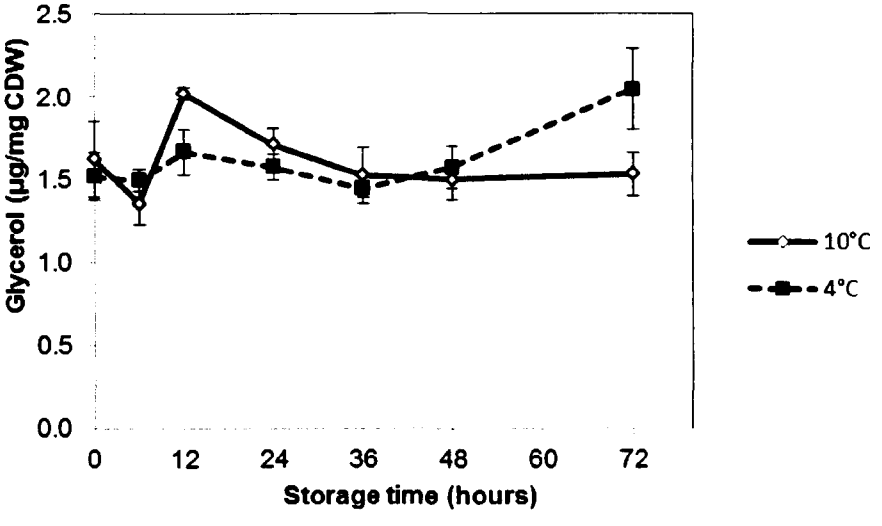



**Figure 6.6 - Intracellular brewing yeast glycerol content following 72 hrs of anaerobic storage at 10°C and 4°C under static conditions. Final values have been represented as µg glycerol released per mg of slurry dry weight (SDW). Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

The regulation of intracellular glycerol levels was based on the transcript profiles of 14 ORFs involved in glycerol's biosynthesis (GO0006114), catabolism (0019563), general metabolism (0006071) and transport (GO0015793). Each gene name and putative function has been listed in Table 6-4. Both *GPD1* and *GPD2*, whose product glycerol-3-phopsphate dehydrogenase converts dihydroxyaxetone phosphate (DHAP) into the glycerol precursor glycerol-3-phopshate (G-3-P) (Albertyn *et al.*, 1994c, Ansell *et al.*, 1997) were repressed in the first 6 hrs of anaerobic storage (Figure 6.7a). After 48 hrs, *GPD1* transcript levels were deemed to be expressed at stable levels whereas *GPD2* was induced by 1.4 fold. Glycerol-3-phosphatase encoding *HOR2* and *RHR2* (Pahlman *et al.*, 2001) demonstrated increased transcript abundance throughout cropped yeast storage (Figure 6.7a). When compared to the start of storage, *HOR2* mRNA content was 5 times higher after 48 hrs whilst *RHR2* was induced by 2 fold. In yeast, glycerol could be dissimilated back into dihydroxyacetone phosphate and fed back into the central glycolytic pathway by two different mechanisms. One mechanism involves the combined action of glycerol dehydrogenase (GCY1p; converts glycerol into dihydroxyacetone) and dihydroxyacetone kinases (DAK1/2p: converts dihydroxyacetone into DHAP) whilst the other comprises of GUT1p (catalyzes glycerol to G-3-P conversion) and GUT2p (converts G-3-P to DHAP) (Pavlik *et al.*, 1993, Ronnow and Kielland-Brandt, 1993, Norbeck and Blomberg, 1997, Blomberg and Adler, 1989). Of the differentially regulated genes, *GCY1, DAK2, GUT1* and *GUT2* were all repressed during storage of cropped W34/70 at 4 °C (Figure 6.7b). Of the glycerol transporters, *STL1* was heavily downregulated whilst *FPS1*, associated with glycerol efflux, was

marginally induced (Ferreira *et al.*, 2005) (see Figure 6.7c). *DGA1* whose product catalyzes the terminal step of triacylglycerol formation (Oelkers *et al.*, 2002) was induced by 1.3 fold after 48 hrs of cold incubation (Figure 6.7c).

**Table 6-4 - List of genes associated with the biosynthesis (GO0006114), catabolism (GO0019563), general metabolism (GO0006071) and transport (GO0015793) of glycerol. Descriptions for gene ontologies (GO) and gene functions were obtained following those of the *Saccharomyces* Genome Database (SGD). (Hong EL *et al.*, 2007 , Engel *et al.*, 2010, Christie *et al.*, 2009).**

| Gene | Description |
|---|---|
| ***GPD1*** | glycerol-3-phosphate dehydrogenase, involved in glycerol synthesis |
| ***GPD2*** | NAD-dependent glycerol 3-phosphate dehydrogenase |
| ***HOR2*** | DL-glycerol-3-phosphatases involved in glycerol biosynthesis |
| ***RHR2*** | DL-glycerol-3-phosphatase isoform; involved in glycerol biosynthesis |
| ***GCY1*** | NADP(+) coupled glycerol dehydrogenase |
| ***DAK1*** | Dihydroxyacetone kinase |
| ***DAK2*** | Dihydroxyacetone kinase |
| ***GUT1*** | Glycerol kinase, converts glycerol to glycerol-3-phosphate |
| ***GUT2*** | Mitochondrial glycerol-3-phosphate dehydrogenase |
| ***GUP1*** | Plasma membrane protein; involved in glycerol transport |
| ***GUP2*** | Probable membrane protein; possible role in glycerol proton symport |
| ***FPS1*** | Plasma membrane channel, involved in efflux of glycerol |
| ***STL1*** | Glycerol proton symporter of the plasma membrane |
| ***DGA1*** | Diacylglycerol acyltransferase |

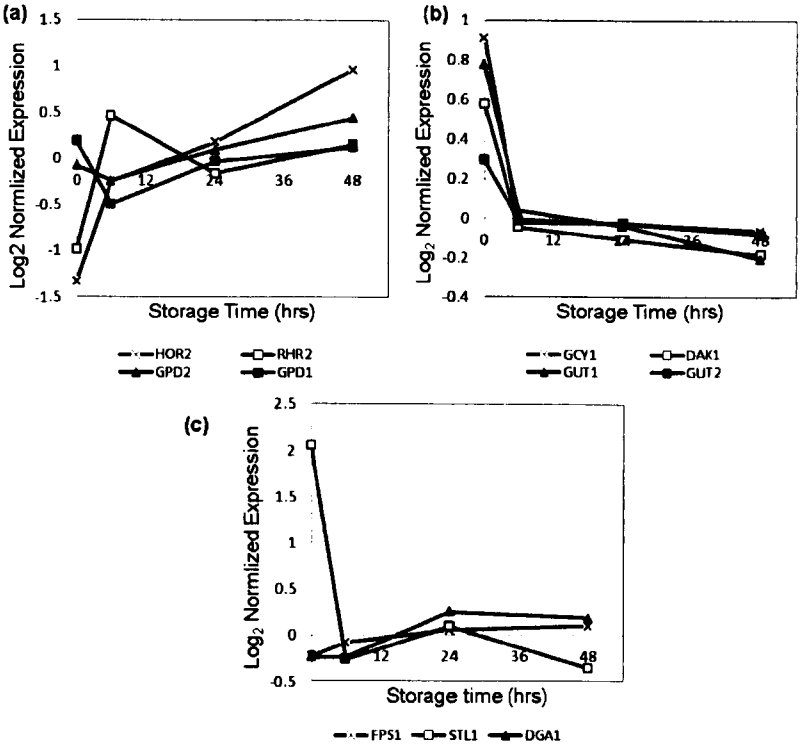



**Figure 6.7 - Transcription profiles of genes associated with the biosynthesis (A), catabolism (B), general metabolism and transport (D) of glycerol during W34/70 fermented yeast slurry storage at 4 °C. All presented genes show a statistically significant change in expression of $p < 0.01$ (one-way ANOVA with BH-FDR correction) and fold change of > 1.3. Data points represent the mean of three per-gene normalized transcription values.**

Increasing the storage temperature from 4 °C to 10 °C, resulted in considerable repression of genes involved in glycerol biosynthesis (*HOR2* and *RHR2*) with concomitant induction of ORFs associated with glycerol dissimilation (*GCY1* and *GUT1*) (Table 6-5). The use of a higher storage temperature also resulted in lower transcript abundance of the glycerol transporter-encoding *FPS1*. All the above changes were confined to the first 6 hrs of storage and transcriptional regulation of the glycerol metabolic

machinery seemed independent of the storage temperature in the subsequent stages of cropped yeast storage.

**Table 6-5 - Fold change in the levels of glycerol metabolism genes in *S.pastorianus W34/70* during 48 hrs of anaerobic slurry maintenance at 10 and 4 °C. Data points represent the mean of three per-gene normalized transcription values. All genes presented show a statistically significant change in expression ($p < 0.01$) and a fold change cut off ≥ 10%. Red bars indicate up-regulation whilst green bars represent downregulated genes.**

| Genes | Fold Change - 10°C vs 4°C | | |
|---|---|---|---|
| | 6 Hrs | 24 Hrs | 48 Hrs |
| *HOR2* | 2.1 | - | - |
| *GUT1* | 1.3 | - | - |
| *RHR2* | 2.5 | - | - |
| *FPS1* | 1.5 | - | - |
| *GCY1* | 2.2 | - | - |

### 6.2.1.5 Changes in intracellular fatty acid distribution

The percentage distribution of constituent intracellular fatty acids in yeast populations stored at the two different temperatures is shown in Table 6-6. Throughout the duration of storage at both 10 and 4 °C, the predominant fatty acid residues were the long chain fatty acids of the C16 species namely palmitic (C16:0) and palmitoleic (C16:1) acids, followed by stearic (C18:0), oleic (C18:1), capric (C10:0), myristic (C14:0) and lauric (C12:0) acids (see Table 6-6). C16:0 comprised of more than 50% of the cellular fatty acid pool and together with other long-chain saturated fatty acids (C14:0 and C18:0) contributed 68 - 70% to the cellular FA composition. Of the medium-chain fatty acids (MCFA), C10:0 was present in higher proportions than C12:0, the latter being present in extremely minute quantities at both temperatures (Table 6-6).

The total long-chain saturated fatty acid (SFA) content of brewing yeast slurry was influenced by the duration of storage but was independent of storage temperatures utilized in the current study (Figure 6.8a). Brewing slurries demonstrated a gradual, but marginal, increase in SFA content in the early (12hrs) and middle (24 and 36 hours) stages of storage (Figure 6.8a). An increase in storage temperature resulted in a slight but significant increase in palmitic acid (C16:0) levels after the first 6 hrs of storage, although this trend was not observed during more prolonged storage (Table 6-6). Neither C14:0 nor C18:0 content seemed to be affected by an increase in the storage temperature (Table 6-6).

The total long-chain unsaturated fatty acid (UFA) fraction in yeast slurries was not influenced by the storage temperature and was predominantly dependent upon the length of storage. A minor decline in UFA content was observed under both storage regimes (Figure 6.8b). After 24 hours, both of the constituent UFAs (and thus the overall UFA content) were significantly higher at 10°C compared to 4 °C, albeit this was not sustained in any of the later stages of cold incubation (Figure 6.8b). At the end of cold incubation, the proportion of C16:1 was similar to that preceding storage whilst C18:1 was diminished in both sets of cropped slurries (Table 6-6).

To obtain a holistic view of variations in individual long chain FAs and its potential impact over plasma membrane fluidity, the yeast membrane unsaturation index (UI) and % medium chain fatty acid (MCFA) content were calculated (Section 2.14, Chapter 2). In general, there was a slight but not statistically significant decrease in UI at both temperatures throughout storage (Figure 6.8c). UI was largely unaffected by the storage temperature. The MCFA

content in 10 °C stored slurry was significantly ($p < 0.05$) lower than 4°C-stored populations after 24 hr of storage (Figure 6.8d). Henceforth, a slight increase was observed, final MCFA levels being similar in the two sets of yeast populations.

**Table 6-6 - Fatty acid (FA) distribution in *W34/70* cell populations (1 x $10^9$ cells) stored anaerobically at 10 °C and 4 °C for 72 hours without any agitation. Data is represented as a percentage of the total fatty acid and has been shown as mean ± standard deviation of three independent replicates.**

| Time (Hrs) | FA - 10:0 | | FA - 12:0 | | FA - 14:0 | | FA - 16:0 | | FA - 16:1 | | FA - 18:0 | | FA - 18:1 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C |
| 0 | 4.0 ± 0.03 | 3.8 ± 0.10 | 0.7 ± 0.00 | 0.7 ± 0.01 | 1.3 ± 0.01 | 1.2 ± 0.03 | 53.5 ± 0.22 | 53.5 ± 0.11 | 17.8 ± 0.15 | 17.6 ± 0.25 | 13.9 ± 0.09 | 14.5 ± 0.51 | 8.8 ± 0.05 | 8.8 ± 0.09 |
| 6 | 3.8 ± 0.10 | 3.9 ± 0.09 | 0.7 ± 0.02 | 0.7 ± 0.01 | 1.2 ± 0.04 | 1.2 ± 0.04 | 53.4 ± 0.09* | 53 ± 0.27* | 17.6 ± 0.30 | 17.6 ± 0.15 | 14.5 ± 0.35 | 14.8 ± 0.39 | 8.8 ± 0.05 | 8.9 ± 0.14 |
| 12 | 3.3 ± 0.21 | 3.7 ± 0.19 | 0.6 ± 0.03 | 0.6 ± 0.03 | 1.0 ± 0.00 | 1.1 ± 0.12 | 54.1 ± 0.49 | 54.1 ± 0.53 | 18 ± 0.26 | 18.3 ± 0.65 | 14.5 ± 0.34 | 13.7 ± 0.88 | 8.5 ± 0.05 | 8.4 ± 0.15 |
| 24 | 3.1 ± 0.10* | 3.7 ± 0.32* | 0.6 ± 0.03 | 0.6 ± 0.04 | 1.0 ± 0.04 | 1.1 ± 0.01 | 53.9 ± 0.30 | 54.8 ± 0.45 | 18.2 ± 0.02* | 17.8 ± 0.19* | 14.5 ± 0.45 | 14.0 ± 0.54 | 8.8 ± 0.12* | 8.0 ± 0.46* |
| 36 | 3.3 ± 0.08 | 3.5 ± 0.14 | 0.6 ± 0.02 | 0.6 ± 0.05 | 1.0 ± 0.07 | 1.1 ± 0.05 | 54.4 ± 0.54 | 54.2 ± 0.59 | 17.7 ± 0.33 | 17.9 ± 0.41 | 14.5 ± 0.73 | 14.0 ± 0.62 | 8.4 ± 0.13 | 8.5 ± 0.27 |
| 48 | 3.5 ± 0.13 | 3.7 ± 0.11 | 0.6 ± 0.08 | 0.6 ± 0.06 | 1.0 ± 0.02 | 1.1 ± 0.02 | 55.2 ± 0.21 | 54.9 ± 0.32 | 17.9 ± 0.53 | 18.1 ± 0.17 | 13.7 ± 0.74 | 13.6 ± 0.24 | 8.1 ± 0.26 | 7.9 ± 0.15 |
| 72 | 3.5 ± 0.24 | 3.8 ± 0.18 | 0.6 ± 0.07 | 0.6 ± 0.07 | 1.1 ± 0.03 | 1.1 ± 0.06 | 55 ± 0.55 | 55.3 ± 0.06 | 17.9 ± 0.53 | 17.8 ± 0.31 | 13.8 ± 0.67 | 13.6 ± 0.57 | 8.2 ± 0.40 | 7.7 ± 0.45 |

* indicates significant difference ($p < 0.05$) between means at 10°C and 4°C analyzed using one way ANOVA; Total FA indicates the overall fatty acid content in µg / mg of cell dry weight.

C10:0 - Capric acid, C12:0 - Lauric Acid, C14:0 - Myristic Acid, C16:0 - palmitic acid; C16:1 - palmitoleic acid; C18:0 - stearic acid; C18:1 - oleic acid.

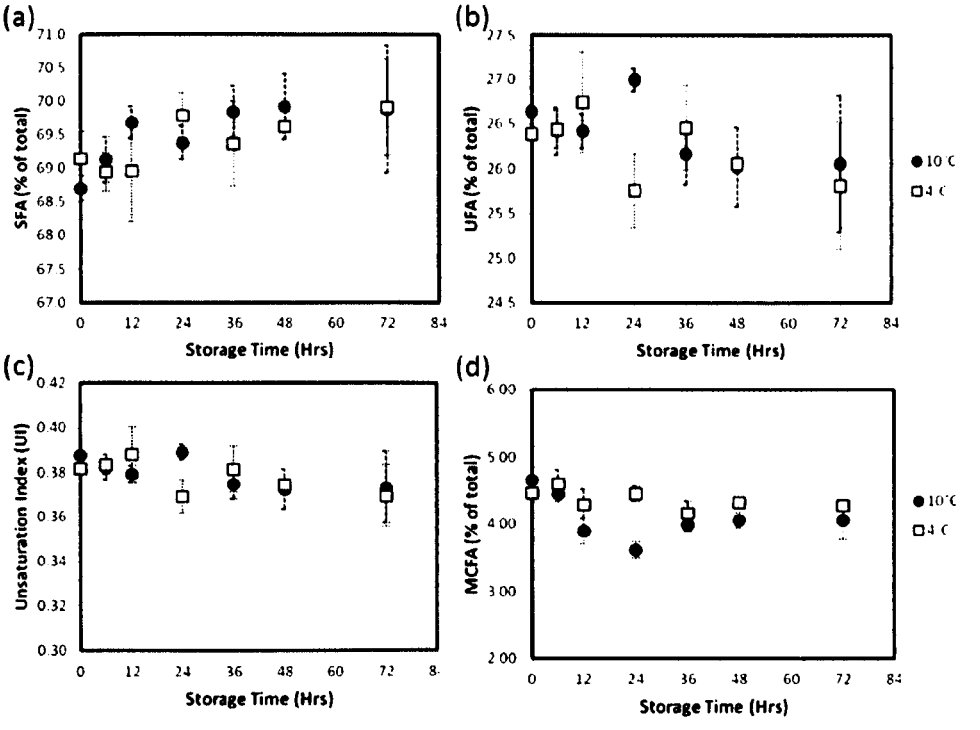



**Figure 6.8 - Alterations in long chain saturated (SFA; a) and unsaturated (UFA; b) fatty acids, unsaturation index (UI; d) and medium chain fatty acids (MCFA; c) in *W34/70* populations maintained at 10°C and 4°C under anaerobic, static conditions for 72 hours. SFA - ∑(C14:0 + C 16:0 + C18:0); UFA - ∑(C16:1 + C 18:1); UI - UFA / SFA; MCFA - ∑(C10:0 + C 12:0).**

The key genes involved in the various fatty acid related pathways in yeast have been listed in Table 6-7. De novo fatty acid synthesis in yeast is carried out by the type I fatty acid synthetase (FAS) enzymatic complex (composed of two non-identical, multifunctional subunits, α and β,organized as a hexamer) in the cytosol and the type II FAS system in the mitochondria (Trotter, 2001, Tehlivets *et al.*, 2007, Beopoulos *et al.*, 2011). Both utilize acetyl-CoA as the initial biosynthetic unit whilst malonyl-CoA acts as the elongation unit by providing two carbons at each step of the growing fatty acid chain. *ACC1*(or *FAS3) / BPL1* (or *ACC2)* (Roggenkamp *et al.*, 1980, Alfeel *et*

*al.*, 1992, Cronan and Wallace, 1995) and *FAS1* (Schuller *et al.*, 1992) which encode for acetyl-CoA carboxylases and the pentafunctional β subunit of the type I FAS complex (Tehlivets *et al.*, 2007) were not significantly expressed during cropped yeast storage at 4°C; *FAS2* (Schuller *et al.*, 1992) which encodes for the trifunctional α subunit demonstrated marginal induction (Figure 6.9A). Unlike type I FAS-mediated cytosolic FA synthesis where the individual functions are carried out by discrete domains on one of the two (α or β) FAS subunits, mitochondrial FA synthesizing type II FAS system harbours the individual enzymatic activities on separate polypeptides (Tehlivets *et al.*, 2007, Beopoulos *et al.*, 2011). Most of the genes encoding different elements of the type II FAS system including *HFA1*, *ETR1*, *CEM1*, *ACP1* and *OAR1* (Hoja *et al.*, 2004, Torkko *et al.*, 2001, Harington *et al.*, 1993, Schneider *et al.*, 1997, Stuible *et al.*, 1998) were down-regulated during cropped W34/70 storage at 4 °C (see Figure 6.9A). Genes involved with the elongation of the synthesized fatty acid precursors including *ELO1*, *FEN1* (or *ELO2*), *SUR4* (or *ELO3*) (Oh *et al.*, 1997, Schneiter *et al.*, 2000) and *TSC13* (Kohlwein *et al.*, 2001) demonstrated peak expression in the initial storage stages followed by repression (Figure 6.9B). The Δ9-desaturase encoding *OLE1* (Stukey *et al.*, 1990) did not show significant change in expression during any of the storage stages. The lack of any consistent induction of biosynthetic, elongation and desaturation genes suggest cellular reluctance towards de novo SFA or UFA synthesis, both in the cytosol and mitochondria.

Besides the internal FA pool, model yeast is known to uptake extracellular FAs from across the plasma membrane either by simple diffusion (depending upon the length and unsaturation state of FA) or via an active

process mediated by proteins of the fatty acid transport (FAT) family (Tehlivets *et al.*, 2007, Trotter, 2001). Transcript abundance of both *FAT1* and *FAT2/PCS60* was significantly increased during slurry storage (Figure 6.9C) suggesting that the cells were equipping themselves for assimilating any available exogenous FAs.

The catabolism or oxidation of both exogenous and endogenous FAs is preceded by an acyl CoA-synthetase mediated FA activation step resulting in the generation of a coenzyme A (CoA) derivative. The mRNA content of *FAA3* and *FAA4*, whose products (along with *PXA1*) catalyse the activation of exo- and endo-genous FAs respectively (Johnson *et al.*, 1994), were increased during W34/70 slurry storage at 4 °C (Figure 6.9C). Degradation or oxidation reactions of saturated fatty acyl CoAs is reminiscent of those of FA synthesis and entails a four-reaction sequence (1- dehydrogenation, 2-hydratation, 3-dehydrogenation and finally 4-thiolytic cleavage) resulting in a two carbon shortening of the FA chain (Beopoulos *et al.*, 2011). Whilst *FOX1* (involved in reaction 1) (Dmochowska *et al.*, 1990) and *FOX2* (reactions 2 & 3) (Hiltunen *et al.*, 1992) remained significantly un-regulated at 4°C, *POT1* (reaction 4) (Igual *et al.*, 1991, Einerhand *et al.*, 1991) demonstrated progressive increase in transcript levels (see Figure 6.9D). Other genes involved specifically in UFA degradation (including *DCI1*, *SPS19* and *ECI1*; GO0006635) remained unregulated whilst those encoding other enzymes of the FA oxidation pathway such as *EHD3*, *MDH3* and *TES1* (GO0006635; SGD) were predominantly downregulated, with the exception of *IDP3* induction (Figure 6.9D).

**Table 6-7 - List of key genes involved with fatty acid metabolism in yeast including its biosynthesis, elongation, desaturation, transport, activation and β-oxidation. Gene descriptions were obtained following those of the *Saccharomyces* Genome Database (SGD) (Hong EL *et al.*, 2007 , Engel *et al.*, 2010, Christie *et al.*, 2009).**

| Gene | Description |
|---|---|
| ***ACC1*** | Acetyl-CoA carboxylase |
| ***BPL1/ACC2*** | Biotin:apoprotein ligase |
| ***FAS1*** | Beta subunit of fatty acid synthetase |
| ***FAS2*** | Alpha subunit of fatty acid synthetase |
| ***ACP1*** | Mitochondrial matrix acyl carrier protein |
| ***CEM1*** | Beta-keto-acyl synthase |
| ***OAR1*** | Mitochondrial 3-oxoacyl reductase |
| ***HTD2*** | Mitochondrial 3-hydroxyacyl-thioester dehydratase |
| ***ETR1*** | 2-enoyl thioester reductase |
| ***PPT2*** | Phosphopantetheine:protein transferase (PPTase) |
| ***OLE1*** | Delta(9) fatty acid desaturase |
| ***ELO1*** | Elongase I |
| ***FEN1/ELO2*** | Fatty acid elongase |
| ***SUR4/ELO3*** | Elongase |
| ***TSC13*** | Enoyl reductase involved in VLCFA elongation |
| ***FAA1*** | Long chain fatty acyl-CoA synthetase |
| ***FAA2*** | Medium chain fatty acyl-CoA synthetase |
| ***FAA3*** | Long chain fatty acyl-CoA synthetase |
| ***FAA4*** | Long chain fatty acyl-CoA synthetase |
| ***PCS60/FAT2*** | Peroxisomal protein; binds AMP and mRNA |
| ***FAT1*** | VLCF acyl-CoA synthetase and fatty acid transporter |
| ***ANT1*** | Peroxisomal adenine nucleotide transporter |
| ***DCI1*** | Peroxisomal protein |
| ***ECI1*** | Peroxisomal delta3,delta2-enoyl-CoA isomerase |
| ***FOX2*** | Multifunctional enzyme of the fatty acid beta-oxidation pathway |
| ***POT1/FOX3*** | 3-ketoacyl-CoA thiolase with broad chain length specificity |
| ***POX1/FOX1*** | Fatty-acyl coenzyme A oxidase |
| ***HFA1*** | Mitochondrial acetyl-coenzyme A carboxylase |
| ***PXA1*** | Subunit of a peroxisomal ATP-binding transporter complex |
| ***TES1*** | Peroxisomal acyl-CoA thioesterase |
| ***EHD3*** | 3-hydroxyisobutyryl-CoA hydrolase |
| ***IDP3*** | Peroxisomal NADP-dependent isocitrate dehydrogenase |
| ***MDH3*** | Peroxisomal malate dehydrogenase |

VLCFA – Very Long Chain Fatty Acid

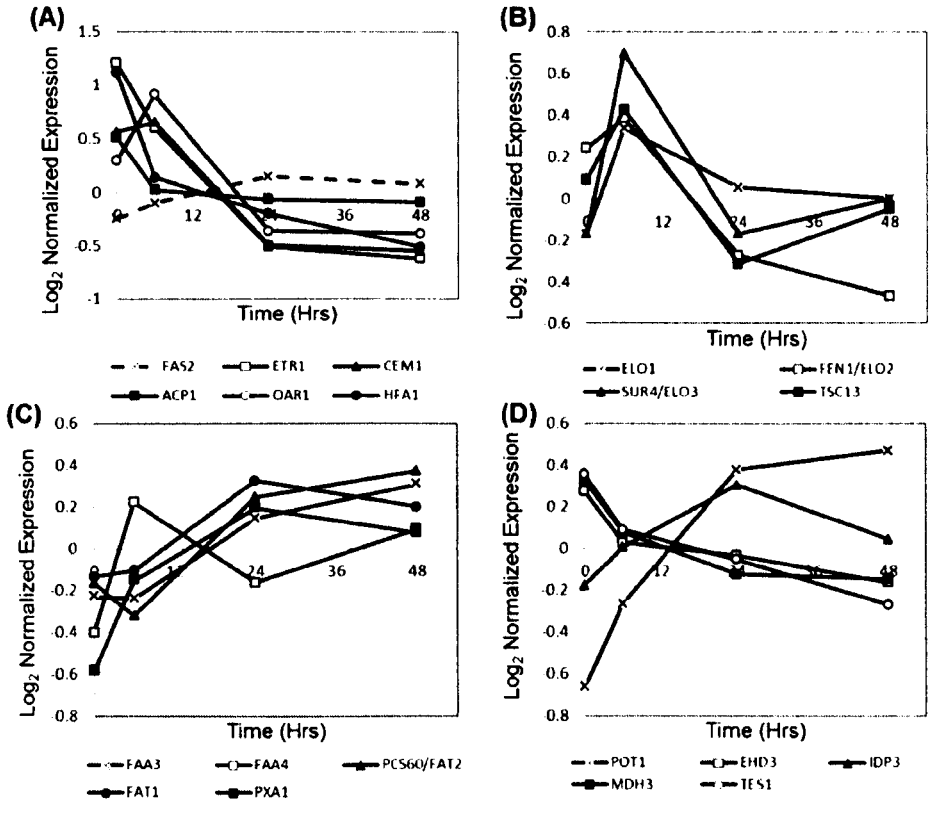



**Figure 6.9 – Transcription profiles of genes associated with the biosynthesis (A), elongation (B), activation, transport (C) and oxidation (D) of fatty acids during W34/70 fermented yeast slurry storage at 4 °C. All presented genes show a statistically significant change in expression of $p < 0.01$ (one-way ANOVA with BH-FDR correction) and fold change of > 1.3. Data points represent the mean of three per-gene normalized transcription values.**

Increasing the storage temperature did not seem to affect the expression of the majority of the genes involved in FA metabolism with only *ELO1* and *ACP1* demonstrating differential transcript numbers in the initial stages of anaerobic cropped slurry storage (refer to Table 6-8).

| Genes | Fold Change - 10°C vs 4°C | | |
|---|---|---|---|
| | 6 Hrs | 24 Hrs | 48 Hrs |
| *ELO1* | 1.5 | - | - |
| *ACP1* | 1.3 | - | - |

**Table 6-8 – Fold change in the levels of glycerol metabolism genes in *S.pastorianus* W34/70 during 48 hrs of anaerobic slurry maintenance at 10 and 4 °C. Data points represent the mean of three per-gene normalized transcription values. All genes presented show a statistically significant change in expression ($p < 0.01$) and a fold change cut off of ≥ 10%. Red bars indicate up-regulation whilst green bars represent down regulated genes.**

## 6.3 Discussion

In the current chapter, the impact of storage temperature on generally recognised key performance indicators of yeast condition were assessed and included: cell viability using citrate methylene violet; budding index; glycogen; trehalose; glycerol and fatty acid composition. An attempt was also made to relate the phenotype with the transcript abundance of the corresponding genes.

### 6.3.1 Effect of cold storage on cell viability and budding index

The most widely accepted indicator of yeast physiological state is viability. In contrast to the low cell viabilities observed during storage of freshly propagated yeast slurries (refer to Chapter 4), G1 cropped *W34/70* populations that had previously conducted one fermentation and were harvested and stored in 2-port storage vessels at 4 °C demonstrated a much higher viability (> 95%) (Figure 6.1). Cropped yeast viability was observed to be independent of storage temperature. These observations partially support those of McCaig and Bendiak (1985b) who had reported similar cell viability maintenance following 2 days of yeast storage at 1, 4 and 10 °C. These authors suggested that prolonged storage at 10 °C yielded poor cell viability, a hypothesis not supported in the current study where longer term storage (3 days) of *W34/70* slurries storage at 10 °C does not seem to cause any marked decline in cell viability (Figure 6.1).

The low Budding Index (BI) (< 2%) in G1 cropped yeast during cold incubation (Figure 6.1) was comparable to earlier values reported by Miller et al (2012). The authors also suggested that lag phase duration in the fermenter is dependent upon the extent of synchrony in the cell cycle positions of

individual cells within the pitching yeast population (Miller *et al.*, 2012). Lack of any significant difference in the BI of stored W34/70 populations thus indicates that cellular synchrony is independent of storage temperature increment to 10 °C and might not affect lag phase duration following re-pitching.

### 6.3.2 Effect of cold storage on yeast glycogen

Optimal glycogen content in cropped yeast is vital for maintaining cellular physiology during cold storage since it fuels yeast basal metabolism in the absence of assimilable nutrients (Quain and Tubb, 1982). Higher brewing slurry storage temperatures have been proposed to accelerate metabolism rate and cellular energy demand resulting in increased degradation of yeast glycogen (McCaig and Bendiak, 1985b). Under the storage conditions employed in current study, W34/70 slurry stored at 10 °C did not significantly alter glycogen mobilization rate (Figure 6.2) and corroborated observations in freshly propagated slurry (Chapter 4). Higher storage temperature prompted an initial transcriptional response towards glycogen accumulation; this was not sustained in later stages (Table 6-2). The expression of glycogen-associated genes largely supported the physiological data and it is proposed that W34/70 glycogen levels are not adversely affected during storage at mildly higher temperatures like 10 °C.

Simultaneous induction of genes associated with both glycogen accumulation and mobilization observed during cropped W34/70 slurry storage (Figure 6.3) mimics the response of *S.cerevisiae* following cold shock (Sahara *et al.*, 2002) (Schade *et al.*, 2004) (Murata *et al.*, 2006), heat shock and oxidative stress (Parrou *et al.*, 1997). In contrast to the proposed recycling of

physiological glycogen in other stresses (Parrou *et al.*, 1997), Schade *et al* (2004) reported considerable accumulation of the carbohydrate following thermal downshift. This was not observed in the current study which differed in the yeast strain, gaseous environment and nutritional availability conditions deployed by Schade *et al* (2004). Thus it is proposed that under anaerobic maintenance, those simulating brewery storage conditions, glycogen accumulation might not be a prerequisite for cold shock adaptation. In support of the above hypothesis, Tai and co-workers (Tai *et al.*, 2007) observed an absence of glycogen accumulation in *S.cerevisiae* chemostat cultures maintained at low temperatures and nutrient limiting conditions.

### 6.3.3 Effect of cold storage on yeast trehalose

Trehalose is a well-recognised stress protectant due to its accumulation during numerous yeast stress responses (Parrou *et al.*, 1997) (Hounsa *et al.*, 1998) (Eleutherio *et al.*, 1993) (Odumeru *et al.*, 1993) (Lillie and Pringle, 1980) (Hottiger *et al.*, 1987) (Schade *et al.*, 2004). Significantly higher levels of the disaccharide were observed in W34/70 populations stored at near-freezing temperatures (Figure 6.4) presumably for cell viability maintenance (Kandror *et al.*, 2002) (Kandror *et al.*, 2004), whilst its presence does not seem imperative for growth at relatively higher temperatures like 10°C (Schade *et al.*, 2004). In the absence of any exogenous carbon source (as indicated by depleted fermentable sugars at the end of G0F; Chapter 5) and extracellular uptake (due to non-significant regulation of transporter *MAL11* in current study) (Plourde-Owobi *et al.*, 2000), trehalose accumulation seemed to occur largely at the expense of glycogen, as suggested in a previous report (Jorgensen *et al.*, 2002).

A significant difference in yeast trehalose as a function of temperature was not reflected at the level of transcriptome regulation (Section 6.2.1.3). Thus, simultaneous activation of both trehalose accumulation and mobilization genes seems to occur under both storage conditions (Figure 6.5). Such disparity between gene expression and cell physiology could partly be explained by the sensitive post-translational control exerted upon most enzymes involved in trehalose (and glycogen) metabolism (Harington *et al.*, 1993) (Huang *et al.*, 1998) (Oh *et al.*, 1997) (Hwang *et al.*, 1989) (Parrou *et al.*, 1999) (Gasch *et al.*, 2000). Co-induction of antagonistic genes can equip brewing yeast for rapid and sensitive modulation of the corresponding enzymes and thus better regulate the carbon flux into and out of its internal reserves (Gasch et al., 2000).

### 6.3.4 Effect of cold storage on yeast glycerol

Although the role of the HOG pathway in yeast osmoregulation and freeze/thaw stress adaptation has been well characterized, evidence suggesting its involvement in cold shock is relatively recent (Wolfe and Bryant, 1999, Panadero *et al.*, 2006). One of the ways that the HOG pathway helps cellular adaptation following temperature downshift is by intracellular glycerol accumulation (Wolfe and Bryant, 1999, Panadero *et al.*, 2006), which was indeed observed in the later stages of brewing yeast storage at 4°C (Figure 6-6). The accumulated glycerol could be directed at $NAD^+$/NADH redox balancing in order to protect cells against freeze injury (Ansell *et al.*, 1997) (Panadero *et al.*, 2006).

Despite simultaneous activation/repression of genes involved in glycerol dissimilation/synthesis during early slurry storage at the higher temperature

(Table 6-5), transient increase in glycerol concentration was observed (Figure 6.6). This is rather surprising since glycerol accumulation in yeast is a direct function of the extent of drop in temperature; higher glycerol levels being reported in *S.cerevisiae* following temperature drop to 4 °C when compared to cellular exposure to 12 °C (Panadero *et al.*, 2006). One reason for such a discrepancy could be lower transcript abundance of the glycerol plasma membrane channel protein encoding *FPS1* during lager yeast storage at 10 °C (Table 6-5). Although *FPS1* is known to be a glycerol facilitator with dual role in both solute uptake and export (Luyten *et al.*, 1995), its physiological role is predominantly in glycerol efflux (Tamas *et al.*, 1999). Decreased *FPS1* activation could thus lead to reduced glycerol efflux eventually resulting in a net intracellular accumulation of the solute. FPS1p mediated glycerol accumulation has been observed under diverse stresses including osmotic (Tamas *et al.*, 1999) and acetic acid stress (Mollapour and Piper, 2007). It is thus plausible that the transient glycerol build-up at the higher temperature is perhaps linked to the observed increase in general cellular stress response during early stages of 10°C – storage (Pahlman *et al.*, 2001) and not necessarily related to temperature variation.

### 6.3.5 Effect of cold storage on plasma membrane fluidity

It is accepted that the composition of the yeast plasma membrane during aerobic and anaerobic conditions is vastly different with saturated fatty acids predominant in the latter (Snoek and Steensma, 2007). This was also reflected in the high SFA presence (68-70% of total) in cropped *W34/70* throughout cold anaerobic storage (Table 6-6 and Figure 6.8a).

The 'homeoviscous' adaptive response (Sinensky, 1974) in yeast aims to compensate for cold stress-induced reduction in membrane fluidity primarily through an increase in the membrane's unsaturation levels or the shortening of its mean fatty acid chain length due to increments in MCFA proportions (Redon *et al.*, 2011). The absence of any obvious difference in UI between *W34/70* populations at 10 and 4 °C (Figure 6.8C) suggests no obvious change in membrane unsaturation probably due to a lack of *OLE1* induction. Consistent MCFA content in 4°C-stored yeast populations (Figure 6.8D) suggests that MCFA increment is also not a favoured route for alleviating any possible membrane rigidification. Thus, changing the storage temperature from 4 to 10 °C did not seem to elicit any obvious physiological adaptation in FA distribution. It is therefore proposed that *W34/70* might be utilizing other mechanisms to maintain membrane fluidity and function. One possibility involves alterations in the membrane's triacylglycerol and phospholipid distribution (Redon *et al.*, 2011). Moreover, increased glycerol at the near-freezing temperature might increase disorder in the distribution of membrane fatty acids (Boggs and Rangaraj, 1985, Beranova *et al.*, 2008) and thus have a direct fluidising effect on plasma membrane flexibility.

It has been suggested that the mRNA levels of FA-synthesizing genes, including *ACC1*, *FAS1* and *FAS2*, peak in a coordinated fashion during the M/G1 phase of cell cycle (Cho *et al.*, 1998, Spellman *et al.*, 1998) whilst expression of genes responsible for FA elongation (such as *FEN1/ELO2* and *SUR4/ELO3*) is down-regulated in stationary phase and under nitrogen depletion conditions (Gasch *et al.*, 2000). This might explain the aversion of non-proliferating, starved W34/70 populations to induce genes involved in FA

synthesis/elongation (Figure 6.9A and B). Instead, cellular efforts were seemingly directed towards inducing genes associated with the uptake of any residual free fatty acids from the extracellular medium (Figure 6.9C). Transcriptomic analysis also provided strong evidence of FA activation (both exo- and endo-genous) and transport into the peroxisomes along with induction of a subset of genes associated with FA oxidation (Figure 6.9C and D). Thus it is tempting to speculate that oxidation of intracellular C18:1 pool along with possible uptake from the extracellular medium might be responsible for the increased C16:0 proportions observed during cold storage of brewing yeast. The fatty-acyl CoAs generated during FA oxidation could potentially be directed into phospholipid formation for membrane fluidity maintenance (Nagura *et al.*, 2004). Further work is required to confirm the validity of the above hypothesis and determine the mechanism(s) associated with membrane stabilization during cold storage of cropped lager brewing yeast slurry.

## 6.4 Conclusion

Maintenance of pitching yeast quality following cropping is vital for the performance and consistency of subsequent fermentations. The data presented in this chapter suggests that cropped *S.pastorianus W34/70* physiology is minimally affected by the increased storage temperature applied, at least for 72 hr storage duration which is widely regarded as exceeding that recommended for good brewing practise (Boulton and Quain, 2001, Briggs *et al.*, 2004). Preservation of glycogen levels at the proposed energy-efficient storage temperature has been proposed as the most important indicator that storage has maintained adequate yeast quality maintenance (Quain and Tubb, 1982). Elevated trehalose and glycerol content towards the end of slurry storage at 4°C might be a manifestation of cellular stress in response to near freezing temperatures. Mimicking propagated yeast (Chapter 4), the FA distribution in G1 cropped yeast which had previously been subjected to a fermentation cycle remained unaffected by the elevated storage temperature. Direct assessment of membrane dynamics, using fluorescent probes such as 1,6-diphenyl-1,3,5-hexatriene (DPH) (Kaiser and London, 1999, Laroche *et al.*, 2001) or trimethylammonium-diphenylhexatriene (TMA-DPH) (Illinger *et al.*, 1989), under slurry storage conditions may provide further insight into brewing yeast behaviour under multiple stresses. Further work is required to investigate the ramifications of elevated storage temperature at production-scale with intermittent agitation (as opposed to no agitation which was applied in the current study). Assessment of a larger cohort of strains encompassing both ale and lager producing yeast is also recommended to ascertain their temperature-dependency under anaerobic storage conditions.

# CHAPTER 7: IMPACT OF HIGHER STORAGE TEMPERATURE ON SERIAL-REPITCHING

## 7.1 Introduction

The quality of pitching yeast is key to the success of brewing fermentations. Not only is the yeast instrumental in the conversion of wort components into alcohol, but a plethora of metabolites generated during fermentation confers the beer its distinct organoleptic profile (Boulton and Quain, 2001, Briggs *et al.*, 2004, Gibson *et al.*, 2007). Maintenance of a steady yeast state during continuous cycles of pitching (inoculation), fermentation, cropping (harvest from fermenter) and storage is critical but not readily achieved and can contribute a major source of variation in the brewing process. Unsatisfactory storage regimes or practises can severely compromise yeast fermentation potential, ultimately leading to process inconsistency in terms of altered residence time in fermenter, attenuation rates, final alcohol values and flavour variations between brews (McCaig and Bendiak, 1985b, Pickerell *et al.*, 1991).

Numerous reports have demonstrated that sub-optimal storage impacts on yeast characteristics including intracellular glycogen, trehalose and lipids and even cell age which in turn effects subsequent fermentation profiles (Sall *et al.*, 1988) (Pickerell *et al.*, 1991) (Guldfeldt and Arneborg, 1998) (Powell *et al.*, 2003) (Verbelen *et al.*, 2009b). However, very few reports addressing the impact of yeast storage temperatures on subsequent fermentations have been published. Murray et al (1984) assessed the effect of storage process conditions, such as the presence / absence of oxygen, temperature and duration, on yeast glycogen and correlated their findings with subsequent fermentation performance. However the authors did not evaluate any of the

other parameters of yeast physiology and the characterization of the ensuing fermentation was rather superficial with an emphasis on yeast cell density and attenuation rates (Murray, 1984) rather than the impact on cellular physiology and beer quality. McCaig and Bendiak (1985) were the first to focus on the impact of storage temperatures on yeast physiology and performed a relatively methodical characterization of subsequent fermentations involving standard measurements such as cell density, attenuation rates, alcohol formation and diacetyl concentrations. However, they did not investigate the potential effects of differential cropped yeast storage temperature on yeast capacity to uptake amino acid and generate flavour metabolites such as higher alcohols, acetate and methyl esters.

Observations concerning the physiology and transcriptome (Chapter 5 and Chapter 6) during anaerobic cropped slurry storage at the traditionally accepted 4 °C and at the proposed 10 °C suggested no obvious deleterious effect on yeast quality at the higher temperature. On the contrary, significantly higher amounts of trehalose and glycerol in populations maintained at the near-freezing temperature indicated towards a stressed phenotype. This chapter addresses the post storage fermentation potential of W34/70 to correlate earlier findings and further evaluate the efficacy of lager brewing yeast storage at 10 °C.

## 7.2 Results

Yeast slurries stored for 72 hours at either 10 or 4°C were pitched into a G1 fermentation (G1F) in mini fermentation vessels (mFVs) (Section 2.10.4, Chapter 2). Cell sampling occurred after 0, 33, 88, 112, 155, 222 and 252 hrs. Fermentation broth was analysed for cell viability (Section 2.11.1, Chapter 2), budding index (Section 2.11.3, Chapter 2) and cell density (Section 2.11.4, Chapter 2) whilst spent wort analysis involved determination of specific gravity and ethanol (Section 2.17, Chapter 2), fermentable sugars (Section 2.18, Chapter 2), free amino nitrogen (Section 2.19, Chapter 2), amino acid content (Section 2.20, Chapter 2), diacetyl (Section 2.21.1, Chapter 2), esters and fusel alcohols (Section 2.21.2 and Section 2.21.3, Chapter 2).

### 7.2.1 Cell Replication and Cell Density

The impact of storage temperature on the cell density profile of yeast during fermentation is shown in Figure 7.1a. The storage temperature before fermentation did not significantly affect the cell density during the early and mid-phases of fermentation, however greater variation was noted towards the end of fermentation. The replicative capacity of yeast in G1F as evidenced by budding index (BI) also appeared unaffected by storage temperature with a maximum BI of 68% and 72% being achieved in fermentations pitched with 10°C- and 4°C-stored yeast respectively (Figure 7.1a). Irrespective of prior storage temperature, the BI dropped to < 10% during mid-fermentation and demonstrated a progressive decline thereafter indicating cessation of cell proliferation. After the first few hours of fermentation, yeast cell viability did not drop below 90% at any of the sampling points irrespective of the storage history of the population (Figure 7.1a).

The rate of utilization of wort fermentable extract, indicated by the fall in specific gravity, is routinely assessed in the brewing industry and is a basic indicator of fermentation progress (Boulton and Quain, 2001, Briggs *et al.*, 2004). Decline in specific gravity during G1F was not affected by storage temperature (Figure 7.1b). Attenuation gravities of 1.8 ± 0.13 and 1.9 ± 0.20 °P were achieved during G1F irrespective of the storage temperature applied to the inoculum. Peak ethanol levels coincided with the end of attenuation gravity during both G1Fs (Figure 7.1b) and neither the rate of ethanol formation nor final ethanol yield were adversely affected by cropped slurry storage at 10 °C.

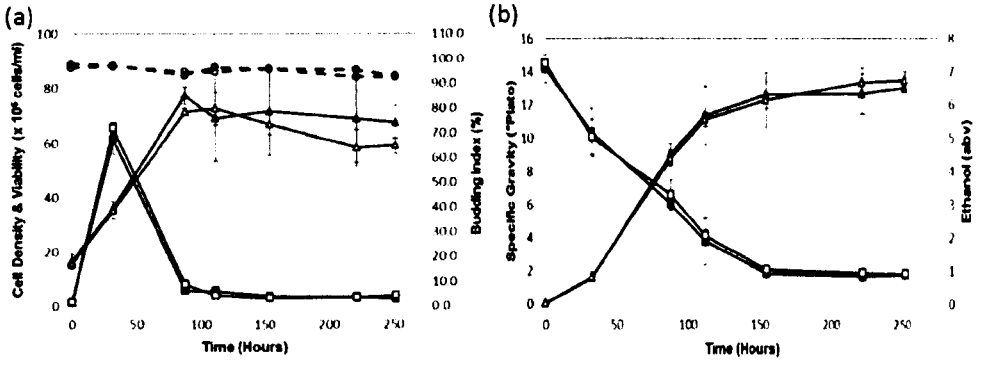



**Figure 7.1 - Key fermentation performance indicators (a) cell density, viability budding index, (b) specific gravity and ethanol during continuously stirred (200 rpm) G1F. Closed and open symbols denote G1F utilizing cropped *S.pastorianus* W34/70 stored at 10°C and 4°C respectively. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

### 7.2.2 Utilisation of wort fermentable sugars

Of the four major fermentable sugars present in wort, maltose is the most abundant whilst fructose is the least (Figure 7.2, graphs a – d). Carbohydrate utilization patterns can sometimes differ depending on the physiological pre-history of the strain (Miller *et al.*, 2012). However, it was observed that inoculum storage temperature did not affect the carbohydrate utilisation profiles of W34/70 during G1Fs ($p > 0.05$). Around 80% of glucose consumption occurred by mid-exponential phase of cell growth during G1F (Figure 7.2a) and this was followed by increased assimilation of the remaining sugars, at substrate specific rates. Complete fructose utilization was observed between mid-logarithmic and onset of stationary phase (Figure 7.2b) along with the consumption of majority of maltose (Figure 7.2c) and maltotriose (Figure 7.2d). The remaining sugar fraction dropped to basal levels after 155 hrs and coincided with attainment of peak ethanol levels.

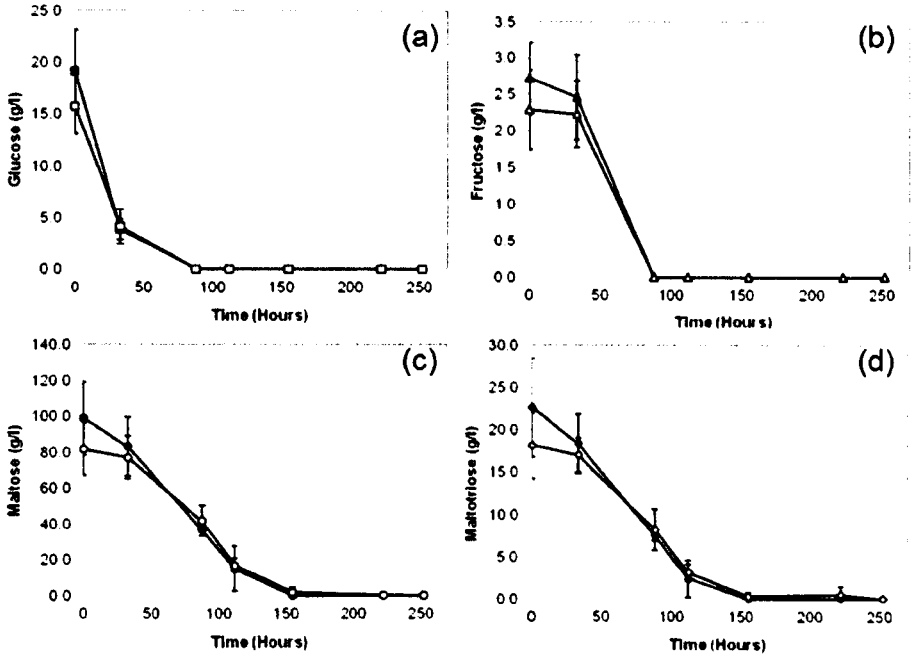



**Figure 7.2 - Residual wort fermentable sugars during continuously-stirred (200 rpm) G1F.Glucose (a), fructose (b), maltose (c) and maltotriose (d) profiles are shown. Closed and open symbols denote G1F utilizing cropped *S.pastorianus* W34/70 stored at 10°C and 4°C respectively. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

### 7.2.3 Utilisation of FAN and Amino acids

The utilisation of free amino nitrogen (FAN) during G1 fermentations was monitored. *W34/70* cropped slurry storage temperature did not appear to affect FAN utilisation (Figure 7.3) ($p > 0.05$). Interestingly not all of the FAN was assimilated during G1F with 40% and 45% left in spent wort. Since amino acids (AA) are the major contributor to wort FAN levels (Ingledew, 1975, Briggs *et al.*, 2004, Gibson *et al.*, 2009) their assimilation patterns during G1F were ascertained.

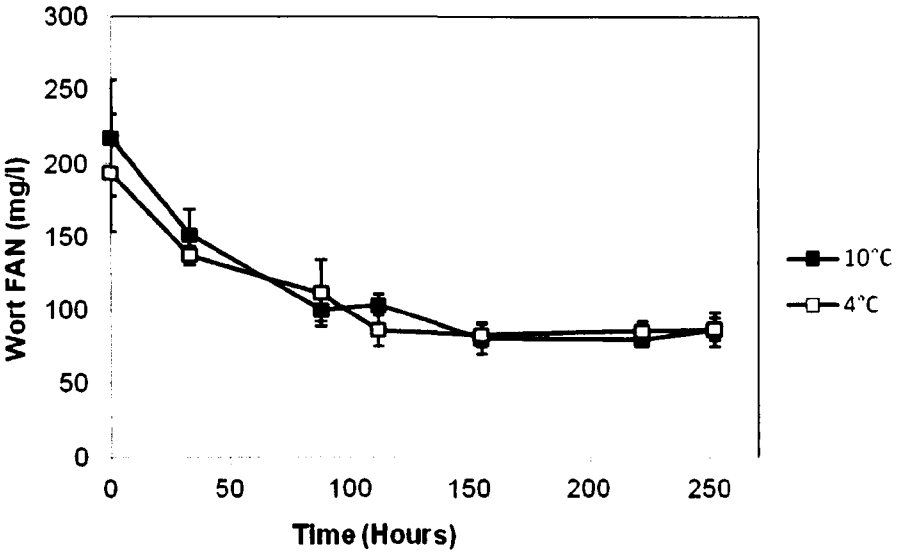



**Figure 7.3 – Free amino nitrogen (FAN) utilisation during G1F pitched with cropped *S.pastorianus* W34/70 populations stored at different temperatures. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

Analysis of the wort used for the G1Fs for the presence of all amino acids (AAs) (except arginine which could not be monitored due to limitations of the assay employed) was completed. Depending on the order and extent of absorption during brewing fermentations, wort AAs have been categorised into four classes – A, B, C and D (Jones *et al.*, 1969, Lekkas *et al.*, 2007). Recent data from a full scale lager fermentation suggests that *Saccharomyces pastorianus* strains may exhibit different uptake profiles altering the constituents of these groups (Gibson *et al.*, 2009). The inoculum storage temperature did not affect the rate or extent of AA assimilation in G1Fs.

Group A AAs were assimilated in early G1F (Table 7-1) with all constituent members of this class (serine, threonine, asparagine, glutamine, lysine, asparate and glutamate) being almost fully depleted after 88 hrs of fermentation (Figure 7.4, graphs A-F and Figure 7.5G). Slight but significant

($p < 0.05$) increases in glutamine and glutamate concentration were observed towards late G1F irrespective of inoculum's storage history. Group B amino acids (methionine, leucine, isoleucine, histidine and valine; Figure 7.5, graphs H - L) were assimilated slowly at the beginning of fermentation (Table 7-1) followed by complete utilization of methionine, leucine and isoleucine after 112 hrs of G1 fermentation. Group C AAs (phenylalanine, tyrosine, alanine, tryptophan and glycine; Figure 7.6, graphs M - Q) demonstrated relatively poor utilization and substantial amounts (46 and 42 % of initial amounts; Table 7-1) remained unassimilated irrespective of the inoculum storage temperature preceding fermentation. Imino acid, proline was not utilized during any of the G1Fs and final levels were insignificantly higher ($p > 0.05$) (Figure 7.6R and Table 7-1). Substantial amounts of total amino acids (43.4 ± 7.7% and 38.1 ± 9.7 %) remained unassimilated at the end of G1 fermentations (Table 7-1) and could have partially, if not majorly, contributed to the high FAN levels observed following G1F completion.

**Table 7-1 – Utilization of amino acids grouped into classes A, B and C in continuously-stirred (200 rpm) G1F pitched with cropped *S.pastorianus* W34/70 slurry stored at different temperatures. Values represent the mean of three independent replicates. The initial concentrations of the various amino acid classes is shown. Absorption has been represented as a percentage of the initial amounts.**

| | [AA] (mmol/L) corresponds to 100% | | % absorption relative to concentration at 0 hrs | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 33 | | 88 | | 112 | | 155 | | 222 | | 252 | |
| | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C | 4°C | 10°C |
| **Class A** | 4.2 | 4.4 | 65 | 66 | 94 | 95 | 97 | 97 | 97 | 96 | 92 | 95 | 93 | 94 |
| **Class B** | 3.3 | 3.6 | 28 | 32 | 63 | 66 | 84 | 76 | 87 | 87 | 86 | 89 | 83 | 80 |
| **Class C** | 3.1 | 3.5 | 11 | 19 | 29 | 35 | 57 | 50 | 58 | 65 | 53 | 65 | 54 | 58 |
| **Total** | 14.1 | 15.0 | 30 | 35 | 43 | 53 | 59 | 55 | 60 | 62 | 58 | 62 | 57 | 62 |

Class A represents the sum total of the concentrations of asparagine, aspartate, glutamate, glutamine, lysine, serine and threonine; Class B represents the sum total of the concentrations of histidine, isoleucine, leucine, methionine, valine; Class C represents the sum total of the concentrations of alanine, glycine, phenylalanine, tryptophan, tyrosine; Total represents the sum total of the concentrations of Proline and amino acids in classes A, B, and C.

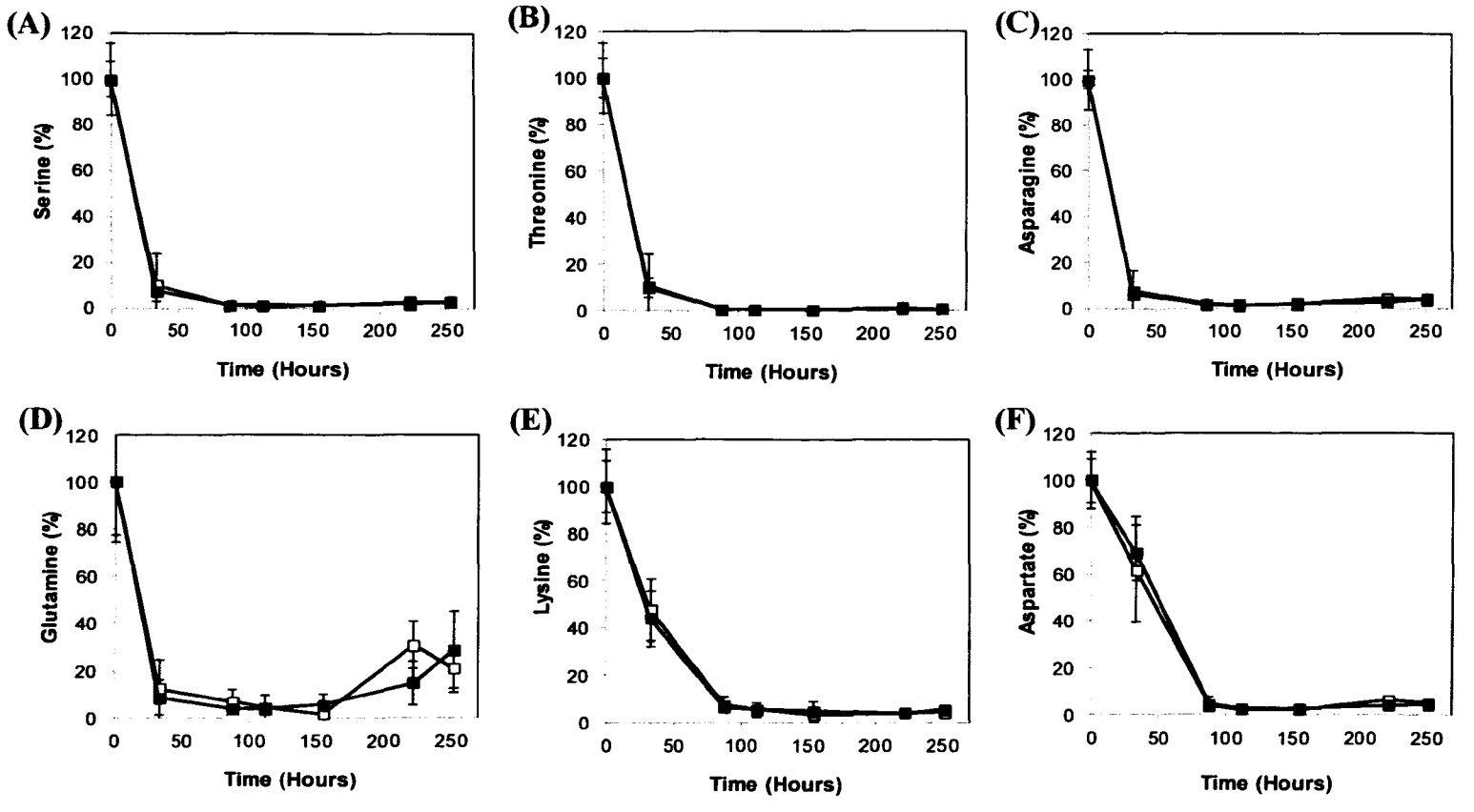



**Figure 7.4 - Utilization of individual amino acids in continuously-stirred (200 rpm) G1F inoculated with *S.pastorianus* W34/70 populations stored at different temperatures. Serine (A), Threonine (B), Asparagine (C), Glutamine (D), Lysine € and Aspartate (F) profiles have been shown. Closed and open symbols denote storage at 10°C and 4°C respectively. Amino acid consumption has been represented relative to their initial amounts (100%). Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

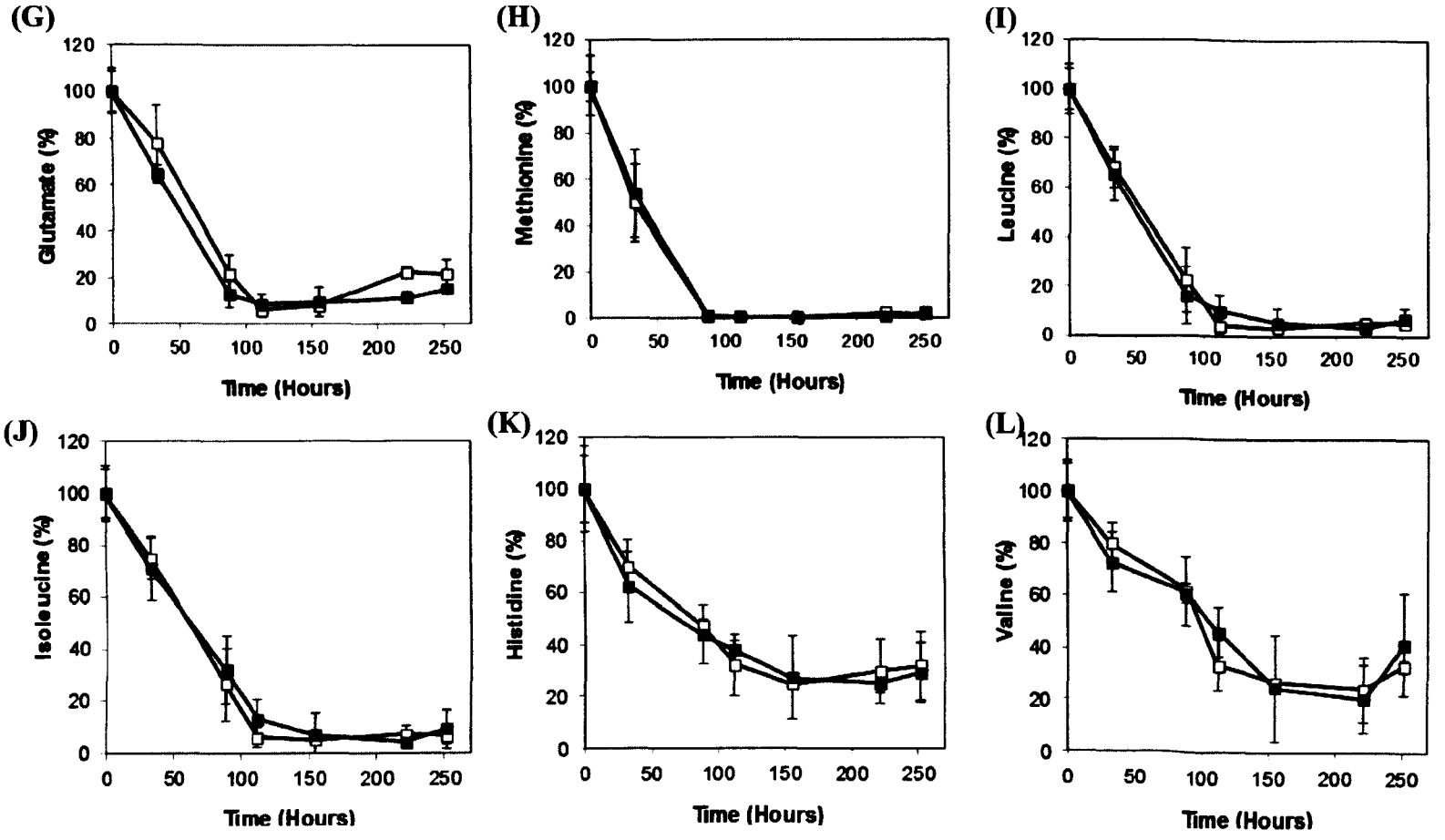



**Figure 7.5 - Utilization of individual amino acids in continuously-stirred (200 rpm) G1F inoculated with *S.pastorianus* W34/70 populations stored at different temperatures. Glutamine (G), Methionine (H), Leucine (I), Isoleucine (J), Histidine (K) and Valine (L) profiles have been shown. Closed and open symbols denote storage at 10°C and 4°C respectively. Amino acid consumption has been represented relative to their initial amounts (100%). Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean (continued frombefore).**

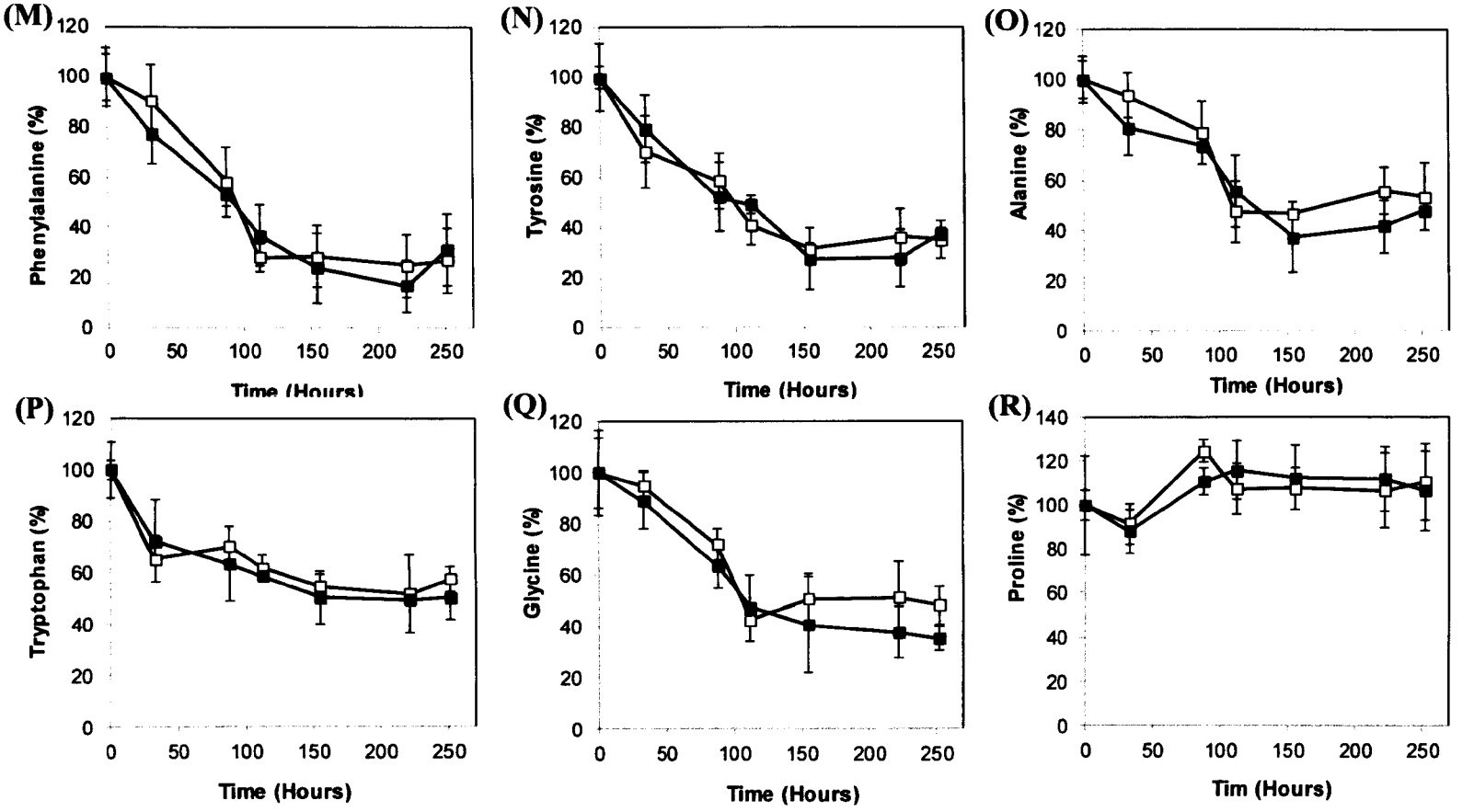



**Figure 7.6 - Utilization of individual amino acids in continuously-stirred (200 rpm) G1F inoculated with S.pastorianus W34/70 populations stored at different temperatures. Phenylalanine (M), Tyrosine (N), Alanine (O), Tryptophan (P), Glycine (Q) and Proline (r) profiles have been shown. Closed and open symbols denote storage at 10°C and 4°C respectively. Amino acid consumption has been represented relative to their initial amounts (100%). Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean (continued frombefore).**

### 7.2.4 Flavour metabolites

The impact of altered storage temperature on flavour profiles was assessed by analyzing the major carbonyl, fusel alcohol and ester compounds in spent wort during the course of G1F using solvent-extraction (Section 2.21.2, Chapter 2) and head space GC-MS (Chapter 2, Section 2.21.3).

#### 7.2.4.1 Carbonyl compounds

Diacetyl (2,3 – butanedione), one of the two vicinal diketones (VDK) formed during valine, leucine and isoleucine synthesis, is a particularly important carbonyl compound in fermenting lager beers. It has a strong "butterscotch" or "toffee" flavour which is deemed unfavourable for overall lager flavour profile (Boulton and Quain, 2001, Verstrepen *et al.*, 2003a). Peak total diacetyl levels during early G1F represents extracellular excretion of acetohydroxy acids followed by spontaneous oxidative decarboxylation to generate diacetyl (Boulton and Quain, 2001, Briggs *et al.*, 2004). Thereafter, diacetyl is re-assimilated and reduced by lager brewing yeast. Change in slurry storage temperature from 4 to10 °C did not cause any significant alteration in G1F total diacetyl profiles ($p > 0.05$) (Figure 7.7a) and end-of-fermentation concentrations remained below its flavour threshold (refer to Table 7-2).

Being the immediate precursor to ethanol and imparting a "grassy" off-flavour, acetaldehyde is a major carbonyl compound (Boulton and Quain, 2001, Briggs *et al.*, 2004). Slurry storage temperature did not result in any significant difference in acetaldehyde levels ($p > 0.05$). Mimicking standard and expected profiles, peak acetaldehyde concentrations were exhibited during early G1F followed by gradual decline (Figure 7.7b) (Boulton and Quain,

2001, Briggs *et al.*, 2004), final concentrations being well below threshold values (Table 7-2).

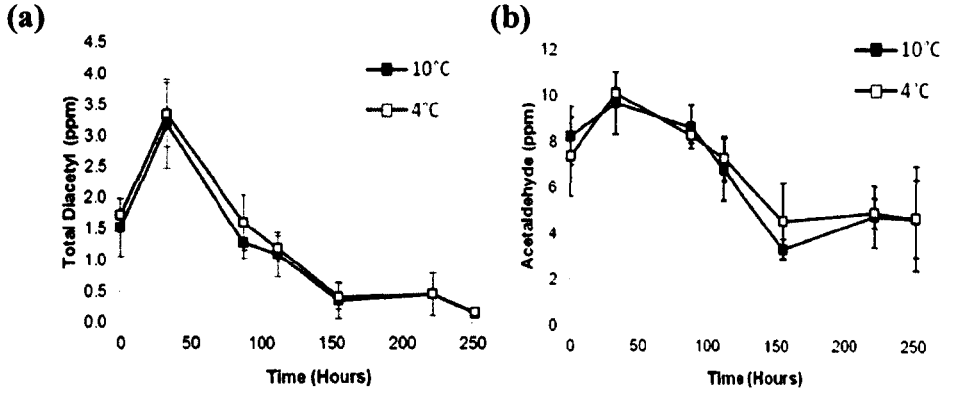



**Figure 7.7 – Total diacetyl (a) and acetaldehyde (b) levels during G1F pitched with cropped *S.pastorianus* W34/70 populations stored at different temperatures. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

### 7.2.4.2 Higher Alcohols

Slurry yeast storage temperatures did not seem to significantly influence the rate of formation and final concentrations of some of the major fusel alcohols during G1F including isoamyl alcohol (3-methyl-1-butanol) (Figure 7.8a), n-propanol (Figure 7.8b), isobutanol (Figure 7.8c) and 2-phenyl ethanol (Figure 7.8d). Higher rates of formation of active amyl alcohol (2-methyl-1-butanol) were noticed in G1F conducted with 10°C-stored pitching yeast (Figure 7.8e) but by the end of fermentation, levels were not significantly different ($p > 0.05$) (Table 7-2). In agreement with typical beer fermentations, G1F employing 10°C-stored yeast exhibited steady fusel alcohol levels once minimal FAN levels were reached (Briggs *et al.*, 2004). There was no apparent lag in the formation of higher alcohols and increased amounts were observed from the first sampling point in both sets of G1F.

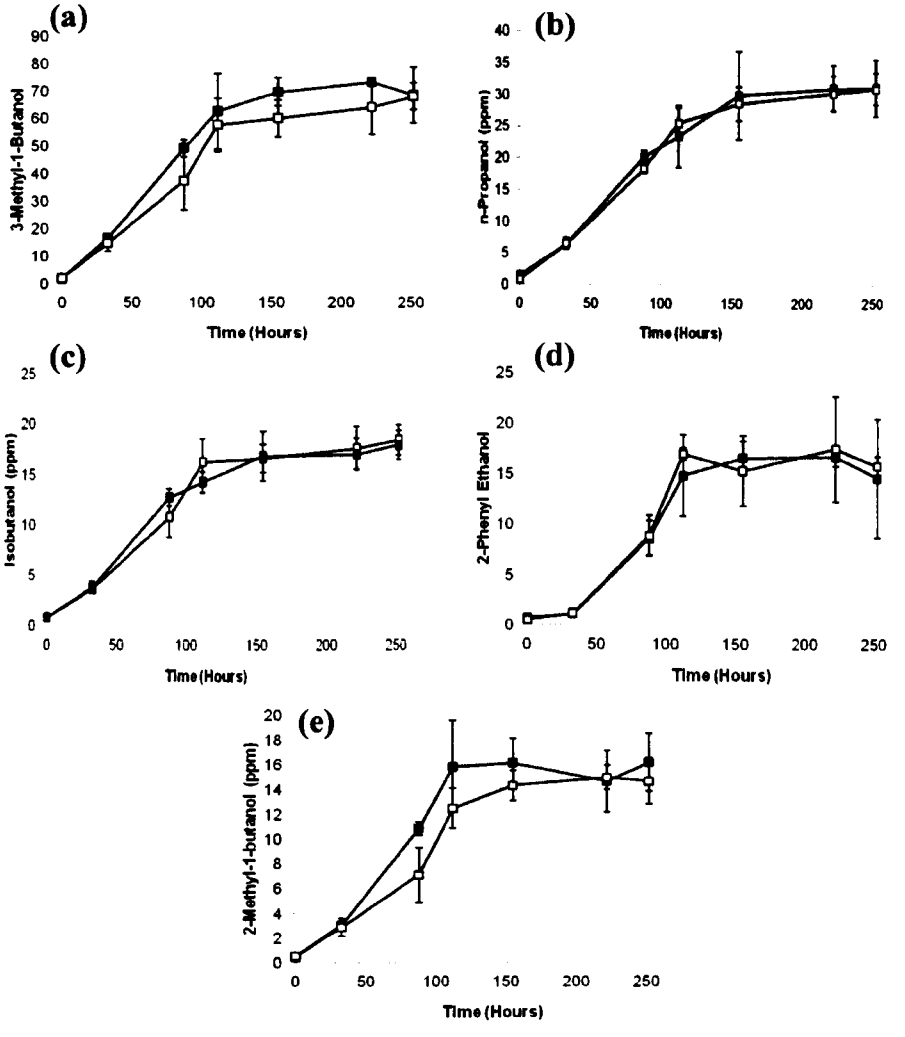



**Figure 7.8 – Major fusel alcohols in continuously-stirred (200 rpm) G1F. 3-Methyl-1-Butanol (a), n-Propanol (b), Isobutanol (c), 2-Phenyl Ethanol (d) and 2-Mehyl-1-Butanol (e) profiles are shown. Closed and open symbols denote G1F utilizing cropped *S.pastorianus* W34/70 stored at 10°C and 4°C respectively. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

### 7.2.4.3 Esters

Aroma-active esters are formed during fermentation and can be broadly classified into the acetate and the fatty acid ethyl esters (Verstrepen *et al.*, 2003a, Boulton and Quain, 2001). The major acetate esters, isoamyl acetate (Figure 7.9a) and ethyl acetate (Figure 7.9b) demonstrated a similar rate of formation and eventual final concentrations in both sets of G1F (Table 7-2).

Slurry storage at the higher temperature seemed to cause elevated 2-phenyl ethyl acetate levels (Figure 7.9c) towards late G1F, although this was not deemed significant ($p > 0.05$). In°reased storage temperature did not seem to effect the formation of any of the assessed ethyl esters such as ethyl octanoate (Figure 7.9d), ethyl propionate (Figure 7.9e) and ethyl butyrate (Figure 7.9f). Esters formation in each G1F was preceded by peak fusel alcohol levels supporting the observations of others with more standard fermentations (Verstrepen *et al.*, 2003a).

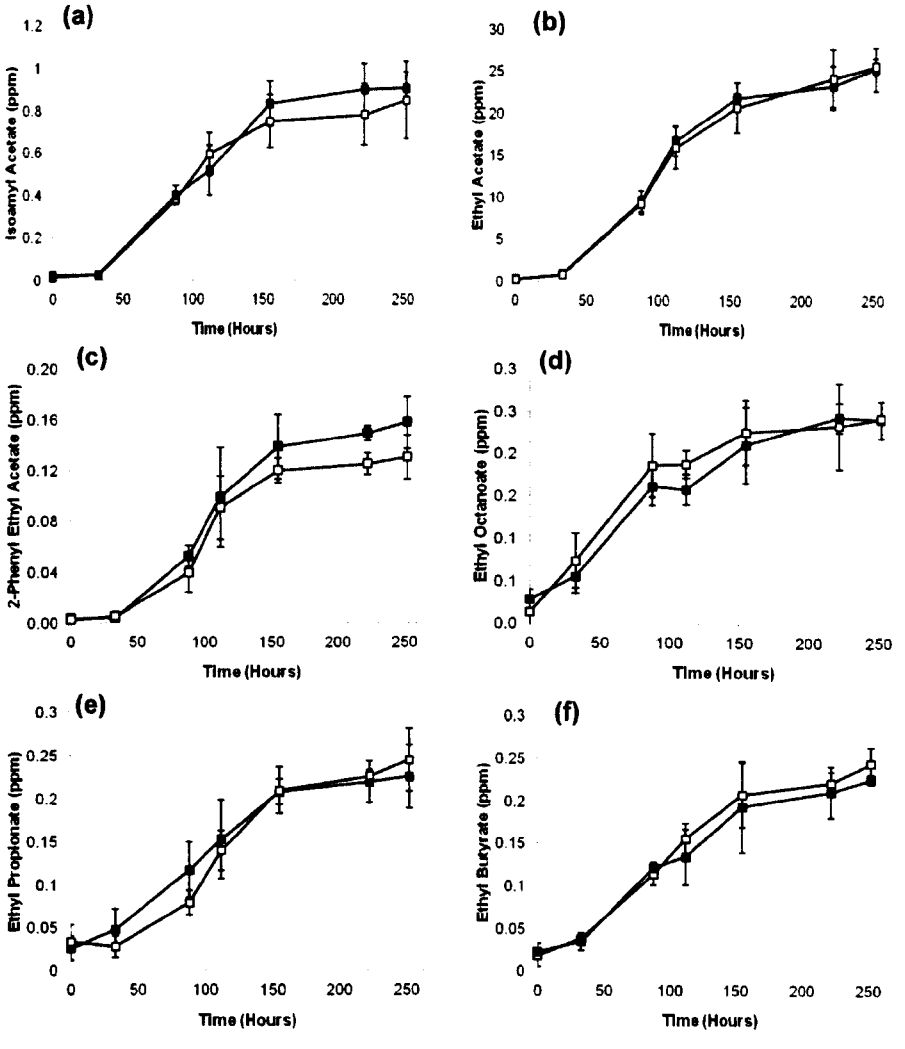



**Figure 7.9 – Key ester compounds in continuously-stirred (200 rpm) G1F. Isoamyl acetate (a), ethyl acetate (b), 2-Phenyl Ethyl acetate (c), ethyl octanoate (d), ethyl propionate (e) and ethyl butyrate (f) profiles have been shown. Closed and open symbols denote storage at 10°C and 4°C respectively. Values represent the mean of three independent replicates and the error bars represent the standard deviation from the mean.**

**Table 7-2 - Concentrations of the major flavour metabolites at the end of G1F pitched with differentially stored W34/70 slurry. The values have been re-calculated to an ethanol percentage of 5.0% and are presented as the mean of three independent replicates along with the standard deviation from the mean.**

| Flavour Metabolite | 10°C-G1F | 4°C-G1F | Threshold* | Range* |
|---|---|---|---|---|
| Diacetyl | 0.1 ± 0.02 | 0.12 ± 0.02 | 0.15 | 0.02 - 0.08 |
| Acetaldehyde | 3.55 ± 1.29 | 3.45 ± 1.68 | 10 | 3.8 - 33.2 |
| **Fusel Alcohols** | | | | |
| 3-Methyl-1-Butanol (isoamyl alcohol) | 52.65 ± 7.78 | 50.56 ± 3.68 | 65 | 32 - 57 |
| 2-Methyl-1-Butanol (active amyl alcohol) | 12.42 ± 1.82 | 10.88 ± 1.39 | 70 | 8 - 16 |
| Isobutanol | 13.71 ± 1.08 | 13.62 ± 1.08 | 16 | - |
| 2-Phenyl-Ethanol | 11.22 ± 0.45 | 11.72 ± 0.75 | 125 | 25 - 32 |
| n-Propanol | 23.93 ± 3.46 | 23.03 ± 1.88 | 800 | 5 - 10 |
| **Esters** | | | | |
| Isoamyl Acetate | 0.7 ± 0.06 | 0.63 ± 0.14 | 1.2 | 0.3 - 3.8 |
| Ethyl Acetate | 19.42 ± 1.96 | 19.01 ± 0.7 | 30 | 8 - 32 |
| 2-Phenyl Ethyl Acetate | 0.12 ± 0.02 | 0.1 ± 0.01 | 3.8 | 0.1 - 0.73 |
| Ethyl Octanoate | 0.18 ± 0.02 | 0.18 ± 0 | 0.9 | 0.04 - 0.53 |
| Ethyl Propionate | 0.17 ± 0.03 | 0.18 ± 0.03 | - | 0.08 |
| Ethyl butyrate | 0.17 ± 0 | 0.18 ± 0.01 | 0.4 | - |

*(Meilgaard, 1975); 10°C-G1F & 4°C-G1F indicates fermentations pitched with yeast slurry stored at 10°C and 4°C respectively.

## 7.3 Discussion

This objective of this chapter was to address the hypothesis that slurry storage temperature would not impact on key performance indicators of fermentation. The hypothesis was developed as a consequence of observations concerning the impact, or lack thereof, of the investigative slurry storage temperatures on the yeast transcriptome (Chapters 5 and 6).

### 7.3.1 Effect of storage on G1F gross cellular characteristics

McCaig & Bendiak (1985b) had observed delayed onset of peak cell densities in fermentations pitched with ale brewing yeast stored at 10 °C. In the current study, the impact of storage temperature on lager brewing yeast cell density and budding index during G1F was investigated (Figure 7.1a). Onset of stationary phase remained unaffected by storage temperatures within the range 4 to 10 °C. Higher storage temperatures have been reported to extend lag phase due to slower cell division in early fermentation on account of poor yeast glycogen content leading to delayed sterol and UFA formation (McCaig and Bendiak, 1985b, Quain and Tubb, 1982, Boulton and Quain, 2001). Although lag phase duration could not be accurately determined due to scarcity of sampling points, cell division was not hampered as indicated by simultaneous onset of peak BI in both sets of G1F (Figure 7.1a). Higher trehalose content in lager pitching yeast has been associated with maintenance of cellular viability during early fermentation (Guldfeldt and Arneborg, 1998). Lower trehalose levels in cropped W34/70 slurry stored at 10 °C did not impair cell viability in G1F (Figure 7.1a) suggesting that reductions in this metabolite to the levels experienced were not deemed a challenge to cell physiology. In accordance with previous reports (McCaig and Bendiak, 1985b), the rate of attenuation,

final specific gravity and ethanol generation remained unaffected by the use of higher storage temperatures (Figure 7.1b).

### 7.3.2 Effect of storage on G1F sugar uptake

One of the major functions of brewery fermentations is the utilization of fermentable sugars in wort to generate ethanol. The pattern of consumption of major fermentable sugars during G1F largely followed convention (Figure 7.2) (Stewart GG, 1983, Gibson *et al.*, 2008), although maltotriose uptake rates were not slower than maltose (Figure 7.2c and d) as would be normally expected (Zastrow *et al.*, 2001). Trans-membrane sugar transport through permeases embedded in the plasma membrane is dependent upon both the degree of fatty acid un-saturation and the levels and composition of sterol in yeast membrane (Watson, 1978, Prasad and Rose, 1986). Structural changes in yeast membrane due to the application of non-optimal storage temperatures have been suggested to affect the action of sugar transporters, such as maltotriose permease, embedded in the membrane and consequent non-utilization of wort fermentable maltotriose (McCaig and Bendiak, 1985b). Similar uptake patterns of wort monosaccharides (glucose, fructose) (Figure 7.2a and b) and α-glucosides (maltose, maltotriose) (Figure 7.2c and d) during G1 fermentations indicates that passive diffusion via hexose transporters (for monosaccharide transport) (Rodrigues de Sousa *et al.*, 2004, Verstrepen *et al.*, 2004) and active sugar transport via the $H^+$-symport mechanism (α – glucoside transport) (Day *et al.*, 2002, Van Leeuwen *et al.*, 1992) are not storage temperature dependent. The rate of carbohydrate utilization was also unaffected by lower trehalose levels in pitching yeast stored at 10 °C, contradicting previous suggestions (Guldfeldt and Arneborg, 1998).

### 7.3.3 Effect of differential storage on G1F nitrogen uptake

FAN which represents the presence of amino acids, ammonia and some end-group α-amino nitrogen in peptides and proteins in wort (ASBC, 1992) was uninfluenced by slurry storage temperature. A decrease in the FAN content of wort occurred until mid-fermentation (Figure 7.3) due to the assimilation of nitrogenous compounds by W34/70 cells for cellular synthesis (Boulton and Quain, 2001). In comparison to ideal end of fermentation FAN levels of 40- 50 mg/l (Briggs *et al.*, 2004), significantly higher proportions (86 ± 11.4 mg/l and 87 ± 7.9 mg/l) remained unutilized following G1F completion irrespective of the pitching yeast storage history. The reasons for this observation are not known. One possible explanation could be that insufficient oxygen availability during the initial stages of fermentation could have limited FAN uptake (O'Connor-Cox, 1993, Verbelen *et al.*, 2009c) .

Amino acid (AA) assimilation has been suggested to directly depend upon yeast quality and storage conditions prior to pitching (Pickerell *et al.*, 1991, Gibson *et al.*, 2009). However, the impact of differential temperature treatment during storage upon AA uptake in ensuing fermentation has not been reported. Increasing the lager brewing yeast slurry storage temperature from 4 to 10 °C did not yield any major difference in the utilization pattern of any of the 18 investigative AAs during G1F Figure 7.4, Figure 7.5 and Figure 7.6). In *S.cerevisiae*, AA transport is governed by two different classes of permease - the general amino acid permease (GAP1) and the high-affinity permeases for transferring individual AAs or a small set of structurally related AAs (Sophianopoulou and Diallinas, 1995, Beck *et al.*, 1999). Thermal downshift affects the levels of GAP1p and several membrane transporters, including two

tryptophan permeases Tat1p and Tat2p (Hernandez-Lopez *et al.*, 2011), the latter also reportedly being degraded during starvation in *S.cerevisiae* (Beck *et al.*, 1999). However slurry storage temperature, did not seem to cause any permanent deterioration in activity of amino acid permeases as evidenced by the similar patterns of amino acid assimilation in both sets of G1 fermentations (Table 7-1).

### 7.3.4 Effect of differential storage on G1F flavour formation

Pitching yeast quality governs the formation of a number of flavour metabolites during fermentation that impart to the beer its characteristic organoleptic profile. Contrary to previous reports (McCaig and Bendiak, 1985b), re-assimilation (and excretion) of total diacetyl seemed unaffected by *W34/70* slurry storage at the higher temperature (Figure 7.7a); however the current study utilized a different brewing yeast strain to these authors suggesting that this response could be strain specific. Poor glycogen content in pitching yeast on account of unsatisfactory yeast handling has been associated with compromised yeast capacity to excrete acetaldehyde in early fermentation (Pickerell *et al.*, 1991). In the current study, no delay in acetaldehyde formation was observed in fermentations pitched with lager yeast populations stored at the higher temperature (Figure 7.7b) thus indicating that adequate amounts of glycogen were maintained, at least until 72 hours of storage.

Yeast produces a number of important long chain and complex alcohols which impart a desirable "warming character" to beer and increase the intensity of ethanol's flavour (Briggs *et al.*, 2004) (Dickinson *et al.*, 2003). Fusel alcohols are derived from the catabolism of branched-chain amino acids (leucine, valine, and isoleucine), aromatic amino acids (phenyl- alanine,

tyrosine, and trytophan), and the sulfur-containing amino acid (methionine) via the Ehlrich pathway (Hazelwood *et al.*, 2008). The latter involves an initial transamination reaction followed by subsequent decarboxylation and reduction, all of which are catalysed by intracellular enzymes encoded by a host of genes (reviewd by (Hazelwood *et al.*, 2008)). *W34/70* populations stored at the higher temperature produced similar final levels of fusel alcohols (Figure 7.8 and Table 7-2) suggesting that storage at 10 °C does not necessarily harm the amino acid catabolism machinery. Despite significantly lower yeast trehalose following incubation at the higher temperature, no significant differences in the rate of formation or final levels of isoamyl alcohol (Figure 7.8a) or isobutanol (Figure 7.8c) were observed in the current study, contradicting earlier reports which differ with respect fermentation temperature and the strain utilised (Guldfeldt and Arneborg, 1998).

In beer, most esters are present at just higher than threshold values (Verstrepen *et al.*, 2003a) and different esters have the potential to act synergistically upon individual flavours (Meilgaard, 1975, Saerens *et al.*, 2008). This implies that slight moderation in their formation during fermentation can impact on the flavour profile of the final product (Verstrepen *et al.*, 2003a) (Briggs *et al.*, 2004). *W34/70* populations stored at 10°C did not seem to yield any significant impact on the formation of major acetate esters - isoamyl acetate (Figure 7.9a) or ethyl acetate (Figure 7.9b) - with final concentrations within the range of previously reported values (Table 7-2) (Meilgaard, 1975). Other flavour-active ethyl esters namely ethyl octanoate, ethyl propionate and ethyl butyrate also seemed unaffected by the inoculum's storage history (Figure 7.9d, e and f). The lag in ester synthesis is in agreement

with previous observations and is probably due to the oxygen mediated repression of the ester synthesising enzymes, especially ATF1p and ATF2p, in early fermentation (Fujii *et al.*, 1996b, Verstrepen *et al.*, 2003b, Ashraf, 2012).

## 7.4 Conclusion

The physiology of pitching yeast dictates the success of brewing fermentations and is key for ensuring beer quality and for maintaining consistency in brewery operations. *W34/70* slurry storage at 10°C did not seem to cause any obvious irregularity in the profiles of any of the key fermentation indicators mentioned in this Chapter. Whilst certain abnormalities in post-storage fermentations were indeed observed (such as reduced FAN and amino acid utilization), this was common to all fermentations and was not due to impaired yeast state on account of slurry storage at the higher temperature. It is proposed that for some lager yeast strains, including *W34/70*, utilization of a higher slurry storage temperature might be acceptable without any compromise on fermentation performance and product quality.

Several (5-10) cycles of yeast storage and repitching is a common occurrence in the brewing industry (Boulton and Quain, 2001, Briggs *et al.*, 2004, Gibson *et al.*, 2007). The current study did not explore the impact of yeast storage at the higher storage temperature on fermentation performance over multiple generations. It is generally accepted that multiple cycles of storage and re-pitching has an adverse effect on yeast quality, particularly for lager strains. This may be manifested in genetic damage (Sato *et al.*, 1994), reduced cell viability (Jenkins *et al.*, 2003), altered flocculation (Smart and Whisker, 1996), aberrant flavour profiles (Watari, 2000) and impaired cell membrane (Jenkins *et al.*, 2003). Thus, further work is recommended for assessing the stability of W34/70 physiology over multiple cycles of storage at the higher temperature and also determining its consequent impact on fermentation performance.

# CHAPTER 8: CONCLUSIONS & FUTURE WORK

## 8.1 Conclusions

The process of serial-repitching in the brewing industry involves collection of yeast biomass at the end of fermentation (termed cropping), followed by storage (for a specific duration) and re-inoculation (termed as re-pitching) into wort for a subsequent fermentation (Boulton and Quain, 2001, Briggs *et al.*, 2004). One of the key stages during serial-repitching which significantly impacts yeast physiology and hence fermentation performance is the environment in which cropped yeast is stored (McCaig and Bendiak, 1985b, Quain, 1988, Heggart *et al.*, 1999) prior to pitching. Key requirements for cropped biomass storage are to maintain yeast in a viable, contamination-free state with good cellular physiology (Heggart *et al.*, 1999). Some of the recommendations for good storage practise include rapid yeast collection at the end of fermentation followed by cellular incubation in a suspension of beer (as cellular slurry) under anaerobic conditions, with intermittent agitation and at low temperatures (O'Connor-Cox, 1997, O'Connor-Cox, 1998, Heggart *et al.*, 1999, Gibson *et al.*, 2007).

Temperature is a key factor that influences yeast state during cropped slurry storage (McCaig and Bendiak, 1985b, Heggart *et al.*, 1999, Boulton and Quain, 2001). Application of high storage temperatures (those exceeding 4 degrees C) have been proposed to be extremely detrimental to yeast physiology (Murray, 1984, McCaig and Bendiak, 1985b). Very few studies have investigated this hypothesis. However, recent work has demonstrated that thermal downshift might impose considerable cold stress on yeast (Sahara

*et al.*, 2002, Schade *et al.*, 2004, Murata *et al.*, 2006) and that tolerance to cold stress may be strain dependent (Leclaire & Smart, In preparation).

The aim of this research was to identify the optimal storage temperature for maintenance of lager yeast W34/70 biomass and to challenge the current industry dogma concerning the requirement to store yeast slurries at near freezing temperatures. A model working system was established to assess the cooling times of W34/70 biomass in different suspension media. This was utilized for investigating the effects of differential temperature treatments on (i) freshly propagated W34/70 (Chapter 4) and (ii) on populations that had experienced a single fermentation cycle (Chapters 5 & 6). The impact of differential storage on subsequent fermentation performance was also evaluated (Chapter 7).

Preliminary investigations focussed on the effect of propagated yeast slurry storage at traditionally accepted recommendations (4 °C) and higher alternatives (10 and 25 °C) on key cellular physiology indicators (Chapter 4). Whilst deterioration in brewing yeast physiology as evidenced by poor cellular viability, proton efflux and impaired glycogen/trehalose content following incubation at 25 °C was in line with expectation, utilization of typically accepted storage temperatures did not preserve yeast physiology to a higher degree when compared to slurry maintenance at 10 °C. However, recycling of spent yeast biomass in the brewery involves storage of yeast slurries after a fermentation cycle. Thus, further experiments addressed the effects of storage temperature increment from 4 to 10 °C in slurries cropped following an initial fermentation (Chapter 5 and 6).

Oligonucleotide microarrays were utilized for genome-scale gene expression analysis (Chapter 5) of yeast populations during storage. Increment in storage temperature stimulated considerable regulation in the yeast transcriptome, although this was predominantly limited to the early stages of storage following which a steady state was seemingly achieved (Chapter 5). Maintenance of lager yeast at the traditionally accepted 4 °C exerted increased cold stress on cell populations, although higher storage temperatures also elicited other unfavourable responses. Amongst the widely accepted genetic markers responsive to cold stress (reviewed by Aguilera *et al* 2007), only *NSR1* demonstrated unequivocal induction and is recommended for indicating the occurrence of cold stress in lager yeast during slurry storage in the brewery. The lack of regulation of other cold-stress indicative biomarkers such as *TIP1*, *TIR1*, *TIR2* and *OLE1*, due to their functional overlap between cold shock and other environmental stresses prevalent during storage, highlights the ambiguity in their accepted status. Mimicking the behaviour of freshly propagated yeast, application of slightly higher temperatures was not considered immensely deleterious to the brewing yeast transcriptome. The microarray results obtained from slurry storage populations were further verified by investigating the impact of storage temperature on cellular physiology (chapter 6) and its fermentation potential (chapter 7).

On account of the dual role of glycogen in providing carbon and energy during cropped yeast storage and for sterol synthesis in initial aerobic phase of subsequent fermentation (Quain *et al.*, 1981, Quain, 1988), preservation of yeast glycogen has been suggested to be the most important indicator suggesting adequacy in cropped yeast storage (Quain *et al.*, 1981, Quain and

Tubb, 1982). Increment in storage temperature from 4 to 10 °C did not lead to any significant change in the rate of glycogen degradation (Chapter 6). Poor glycogen levels in pitching yeast on account of unsatisfactory yeast handling can cause extended lag phase, delayed wort attenuation and compromised yeast capacity to excrete acetaldehyde in early fermentation (Quain and Tubb, 1982, Pickerell *et al.*, 1991), none of which were observed in fermentations pitched with yeast populations stored at the higher temperature (Chapter 7).

The disaccharide trehalose is a well-recognised stress protectant (Majara *et al.*, 1996) and its accumulation, along with glycerol, has been deemed critical for yeast survival at near-freezing temperatures (Kandror *et al.*, 2004, Panadero *et al.*, 2006). This was indeed observed following extended slurry storage at 4 °C and suggests that prolonged maintenance at low temperatures might expose the cropped yeast to considerable environmental stresses (Chapter 6). However, higher trehalose levels in pitching yeast have been suggested to enhance cell viability maintenance in early fermentation, accelerate carbohydrate utilization and increase formation of flavour metabolites such as isobutanol and isoamyl alcohol (Guldfeldt and Arneborg, 1998). None of the aforementioned fermentation characteristics were affected by lower trehalose levels in brewing yeast stored at 10 °C (Chapter 7) suggesting that the storage regime was effective in maintaining optimal trehalose levels necessary for adequate cellular performance in subsequent fermentation.

A plethora of yeast metabolites are produced during fermentation, many of which are flavour active at the concentrations found during fermentation and make a substantial contribution to the taste and aroma of the final product (Verstrepen *et al.*, 2003a, Saerens *et al.*, 2008, Verbelen *et al.*, 2009a). Even

slight modifications in flavour compounds can act synergistically and result in significant changes in beer's organoleptic profile resulting in variation in product quality. A thorough characterization of post-storage fermentations revealed negligible impact of the increased storage temperature on the generation of major flavour metabolites (diacetyl, acetaldehyde, esters and higher alcohols) (Chapter 7) and other key fermentation indicators including sugar uptake, alcohol production and amino acid assimilation (Chapter 7). This further substantiates the efficacy of lager yeast storage at 10 °C. It is therefore proposed that the slurry storage temperature should be tailored to the yeast strain used. Where higher storage temperatures are applied there are obvious benefits in the reduction of energy inputs for slurry storage, providing these do not cause detrimental effects to the yeast and subsequent fermentation performance.

Thermal downshift has been demonstrated to elicit a distinct transcriptional response in haploid laboratory strains of the *Saccharomyces* genus, depending upon the severity of the low temperature and the duration of stress exposure (Sahara *et al.*, 2002, Murata *et al.*, 2006, Al-Fageeh and Smales, 2006, Aguilera *et al.*, 2007). However, the transcriptional response in aneutetraploid strains, such as lager brewing yeast, has not yet been reported. This omission was addressed in this thesis using oligonucleotide microarrays (Chapter 5). Lager brewing yeast, obtained after an initial fermentation, was found to elicit a global genomic response during anaerobic slurry storage at 4 °C in two phases. The early phase was primarily focussed upon de novo ribosome biogenesis and RNA processing presumably to counter the cold-shock mediated inactivation of cellular ribosomes and for unwinding or degradation

of cold-stabilized mRNA secondary structures (Spellman *et al.*, 1998, Zhang *et al.*, 2001, Tehlivets *et al.*, 2007) so as to ensure efficient translation initiation. In the late phase, a coordinated repression of the bulk of the global genome was observed in line with previous reports investigating yeast cold response (Kudo *et al.*, 1996, Murata *et al.*, 2006, Sahara *et al.*, 2002, Schade *et al.*, 2004). From a brewer's point of view, this decrease in cellular activity is the corner stone of preserving yeast viability and fermentation potential during storage. Anaerobiosis and starvation were also found to regulate gene expression during slurry storage in parallel to cold stress.

## 8.2 Future Work

This thesis has focussed on the application of different temperatures during storage of lager brewing yeast slurry at laboratory-scale with no intermittent agitation of stored slurry, as is the convention in brewing industry (O'Connor-Cox, 1997, Heggart *et al.*, 1999, Gibson *et al.*, 2007). Thus it is recommended that further experiments with standard yeast cropping and storage practises be conducted on production-scale.

The application of higher storage temperatures on serial-repitching was limited to a single generation in the current work, as opposed to common brewing practise of repitching for 5-10 generations (Boulton and Quain, 2001, Briggs *et al.*, 2004). Multiple storage and re-pitching cycles is known to have an adverse effect on yeast quality (Sato *et al.*, 1994, Watari, 2000, Jenkins *et al.*, 2003) and higher storage temperatures can only be deployed if cropped yeast from a storage regime utilizing elevated temperatures would be "fit" for subsequent fermentation after multiple generation. .

The use of cylindroconical fermentation vessels has been demonstrated to cause inefficient cooling of the yeast slurry (Cahill, 1999). Thus if higher storage temperatures were to be applied care needs to be taken to avoid formation of thermal gradients in yeast slurry. Cropped yeast storage in the form of pressed cake has been proposed to be more sensitive to storage temperature (Martens, 1986) and determining optimal temperatures for such storage methods also warrants further investigation.

Thermal downshift alters the normally crystalline and fluid plasma membrane into a more rigid gel-phase state, thus prompting an adaptive 'homeoviscous' response to antagonize membrane rigidity (Sinensky, 1974, Thieringer *et al.*, 1998). Irrespective of storage conditions, lager brewing yeast did not increase the levels of membrane unsaturation or reduce the mean length of membrane fatty acids, the two most widespread approaches for increasing membrane fluidity (Thieringer *et al.*, 1998, Redon *et al.*, 2011). Thus direct assessment of membrane dynamics under slurry storage conditions is recommended to determine whether brewing yeast employs alternative mechanisms for maintaining membrane fluidity or concomitant stresses override the effects of cold stress on cell membrane dynamics.

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**Appendix -**

**Statistical Analysis**

Probability ($p$) values were generated following pairwise comparisons between multiple means using one-way Analysis of Variance (ANOVA) with Tukey's honestly significance difference (HSD) correction (Microsoft Excel 2010, SPSS 16.0.0). Unless otherwise stated, $p < 0.05$ was deemed significant at all instances. Normal distribution for the variables was assessed using the One-Sample Kolmogorov-Smirnov Test and group variance on the dependent variable was tested using Levene's Test of Homogeneity of Variance (SPSS 16.0.0). Linear relationship between variables was assessed using Pearson's product moment correlation (SPSS 16.0.0). Statistical analysis on the microarray data was performed using the GeneSpringGX 11 software (Agilent Technologies, USA).